**Functionalized self-assembling peptide hydrogel enhance maintenance of hepatocyte activity in vitro**

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**Abstract**

There is a major challenge in maintaining functional hepatocytes in vivo as these cells rapidly lose their metabolic properties in culture. In this work we have developed a bioengineered platform that replaces the use of the collagen I – in the traditional culture sandwich technique – by a defined extracellular matrix analogue, the self-assembling peptide hydrogel RAD16-I functionalized with biologically active motifs. Thus, after examining side by side the two culture systems we have found that in both cases hepatocytes acquired similar parenchymal morphology, presence of functional bile canaliculi structures, CYP3A2 induction by dexamethasone, urea production, secretion of proteins such as apolipoprotein (class A1, E, J), α1-microglobulin, α1-macroglobulin, retinol binding protein, fibronectin, α1-inhibitor III and biotin-dependent carboxylases. Interestingly, by assessing in more detail some other hepatic markers, one of the functionalized matrix analogues – carrying the 67 kD laminin receptor ligand – enhanced the gene expression of albumin, HNF4-α, MDR2 and tyrosine aminotransferase. We conclude that the use of a synthetic culture system with designed matrix functionalization has the advantage in controlling specific cellular responses.

**Keywords:** extracellular matrix analogue • hepatocyte function • sandwich culture system • self-assembling peptides • collagen • liver enriched genes • real-time PCR

**Introduction**

The liver is a complex organ which performs many basic functions and metabolic activities including synthesis of plasma proteins, storage of glycogen, removal of toxins and metabolism of xenobiotics among others. When isolated, hepatocytes lose their surrounding microenvironment which comprises the extracellular matrix and the interaction with other hepatic cells such as sinusoidal endothelial cells, Kupffer cells, stellate cells, and resulting in a rapid loss of liver-specific functions. Many strategies have been used to obtain in vitro cultures which maintain these hepatocyte functions, including the use of extracellular matrices like Matrigel, collagen or liver derived basement membrane matrix [1–8] or cocultures with hepatic non-parenchymal cells [9–12]. Most recently there has been an approach towards the use of synthetic biomaterials such as hydrogels, porous membranes or the potential utility of perfused bioreactors to assess these goals [13–17].

We previously demonstrated the use of the self-assembling peptide hydrogel Puramatrix (RAD16-I, peptide sequence Ac-RADARADARADARADA-CONH2, R argininge, A alanine, D aspartic acid) for certain tissue engineering applications. This synthetic and defined biomaterial is made of interweaving nanofibres 10–20 nm in diameter, which highly mimics some features of the extracellular matrix. This scaffold has previously been shown to promote the differentiation of a liver progenitor cell line (Lig-8) into hepatocyte-like spheroids [18]. In another study, it was used to entrap hippocampal neural cells [19] and, to enable osteogenic differentiation of mouse embryonic stem cells and mouse embryonic fibroblasts [20]. Moreover, we designed and characterized modified peptide scaffolds with biologically active peptide sequences in order to create instructive synthetic extracellular matrices [21].

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In this work, we have used functionalized self-assembling peptide scaffolds to build a hepatocyte culture system based on the collagen sandwich configuration, considered today as the gold standard for primary hepatocyte culture in vitro [5, 7] which recreates hepatic architecture. Here, we have combined RAD16-I with a modified self-assembling peptide scaffold containing the integrin-binding sequence GRGDSP (RGD with a modified self-assembling peptide scaffold containing the integrin-binding sequence GRGDSP (RGD = Ac-GRGDSP-GG-RADARADARADARADA-CNH2), the 67 kD laminin receptor binding sequence YIGSR (YIG), or the heparin binding sequence present in collagen IV TAGSCLRKFSTM (TAG) in order to create a microenvironment that has features of extracellular matrix in vivo [21]. We have compared the maintenance of some liver specific functions of these sandwich systems to the results obtained using a similar collagen sandwich counterpart. We have analysed several liver-specific functions in terms of gene expression by means of quantitative PCR, examined albumin and urea secretion, and studied several cellular proteins of interest such as albumin, HNF4-α and biotin-dependent carboxylases. We also analysed the secreted proteome in which we detected several plasma proteins and proteases such as apolipoproteins A1, E and J, α1-microglobulin, α1-macroglobulin, retinol binding protein, fibronectin and α1-antitrypsin III. We have found that, hepatocytes cultured in a peptide sandwich configuration maintain similar levels of differentiation as in collagen sandwich cultures, as observed by comparable levels of the liver-specific functions studied. We have also observed that CYP3A2, one of the key Phase-I drug-metabolizing enzymes, can be induced to the same levels of that in collagen sandwiches. This peptide culture system presents the advantage of being completely synthetic and defined. This provides a solution to the problems associated with the use of a naturally derived matrix, including batch to batch deviations and the possible carryover of pathogens. Moreover, peptide sequences can be added to the matrix in a facile manner by direct solid phase synthesis, systematic variation of molecular cues in the extracellular environment and tailoring of that environment to promote specific activities.

Materials and methods

Hepatocyte isolation

Hepatocytes were obtained from male Fischer rats weighing 150–230 g by a perfusion using Liberase Blendzyme 3 solution (Roche, Indianapolis, IN, USA, 1814184). Each isolation yielded hepatocytes with viabilities 75–95% as based on trypan blue exclusion. Hepatocytes were suspended in hepatocyte growth medium (see below) and used for collagen or peptide sandwiched cultures.

Culture of primary hepatocytes in a peptide sandwich system

Collagen (Rat tail collagen, BD Biosciences, San Jose, CA, USA, 354236) substrates were prepared at a final concentration of 2 mg/ml following the manufacturer’s instructions. Briefly, the collagen solution was diluted in water and PBS 10-, and neutralized with 1M NaOH. A total of 80 or 40 μl of the collagen solution were loaded into the bottom of the tissue culture insert (Millipore, Billerica, MA, USA, PICM 1250) and the collagen was allowed to gel at 37°C for 30 min. The 80 μl and 40 μl gels provided 1- and 0.5-mm-thick gels, respectively, and will be referred to by height in the text. After that time, hepatocyte culture medium (HCM, Cambrex, MD, USA, CC-3198, containing 2% fatty acid free bovine serum albumin [BSA], transferrin, insulin, recombinant human epidermal growth factor, ascorbic acid, hydrocortisone and Gentamicin/Ampicillin) was added to the system. Then, 65,000 cells/cm² were loaded into the tissue culture insert. Cultures were incubated at 37°C overnight. The next day, the unattached cells were removed by washing two times with sucrose 10% and another layer (80 or 40 μl) of collagen were added on top and allowed to gel in the incubator for 30 min. After that time culture medium was added to the bottom of the well and inside the insert. Cultures were maintained for 10 days in a water-jacketed incubator at 37°C and 5% CO₂. Culture medium was changed daily.

Culture of primary hepatocytes in a peptide sandwich system

Peptide scaffolds were prepared by diluting a 1% (w/v in water) peptide hydrogel RAD16-I solution (Puramatrix, BD Biosciences, 354250) with an equal volume of 20% sucrose (w/v in water) to achieve final peptide concentration of 0.5% (w/v in 10% sucrose). When the modified peptides were used, they were mixed in a proportion 95:5 with the prototypic peptide (RAD16-I) before dilution with the sucrose solution, vortexed and sonicated for 10 min. to ensure homogeneous mixing. The peptide solution becomes a hydrogel when it contacts a salt solution, such as a buffer or culture media [22]. Thus, 80 or 40 μl volume of peptide was loaded into the bottom of a tissue culture insert (Millipore, PICM 1250), and 200 μl of HCM was added underneath the insert membrane to initiate gel formation, giving rise to 1 and 0.5-mm-thick gels. After the peptide gel was set (10–15 min.), 200 μl of HCM was added into the insert, and the gels were allowed to equilibrate for 30 min. in the incubator at 37°C. The media were then changed and a volume of hepatocyte cell suspension was added to give a final cell density of 65,000 cells/cm² and cultures were incubated overnight to allow cell attachment. The next day, unattached cells were washed by rinsing them three times with sucrose 10%. Then, 80 or 40 μl of peptide were added on top, to entrap the cells in a sandwich fashion. 200 μl of media were added above the insert membrane, and the solution was allowed to gel for 30 min. After that time the peptide was equilibrated by carefully adding 20 μl of media on top, which was then left to sit for 20 min., and then the process was repeated one more time. Finally, a 50 μl volume of medium was carefully added into the insert and 650 μl were added outside of it. The medium was carefully changed daily, to avoid disturbing the upper peptide layer. Cultures were maintained for 10 days in a water-jacketed incubator at 37°C in 5% CO₂.

Bile canicular-like structure staining

Transport in bile canicular-like structure was visualized by treatment of the cultures with fluorescein diacetate (FDA). Briefly, to assess functional transportation by bile canaliculi, media containing 2.5 μg/ml of FDA (Molecular Probes, San Francisco, CA, USA) was added to 7-day peptide
sandwich cultures. Cells were incubated for 20 min. and visualized under a Nikon Epifluorescence microscope (Nikon, Boston, MA, USA).

RNA isolation and qPCR analysis

RNA of days 7 and 10 (not included) cultures was obtained by treating the cells with Trizol Reagent (Invitrogen, Carlsbad, CA, USA, 15596–026), followed by an extraction with an RNaseasy kit (Qiagen, Valencia, CA, USA, 74104). RNA of freshly isolated hepatocytes was extracted and used as a control. The obtained RNA was quantified, the quality was assessed, and then samples were treated with DNase I (Invitrogen, 18068) to remove contaminant DNA. Then, 100 or 150 ng was reverse transcribed with an Omniscript reverse transcription kit (Qiagen, 205111) using random hexamers. Finally, q-PCR was carried out using QuantiTec SYBR Green PCR kit (Qiagen, 204143) in a MJ Opticon Monitor instrument as it has been previously described [23]. The primer sequences were the following: albumin forward 5'-GTTGAGGAATACGACTTGG-3' and reverse 5'-TAACCTGTCTCGAAGAGTGTG-3'; HNF4-α forward 5'-CTGAGACTTCCAGCACTCA-3' and reverse 5'-CTGAGGCTCTCCGCTTGG-3'; CYP3A2 forward 5'-GTATAGCTTCTCTCATTCCACCC-3' and reverse 5'-GTTGCTTATGCTGATACGTCAAC-3'; TAT forward 5'-GATCCTGAGAAACATGGACG-3' and reverse 5'-TTGTACTTCCCAGTCCAG-3'; MDR2 forward 5'-CTTGTGCTGGGAGACACTCT-3' and reverse 5'-CAAGAGAGCCAGCGAACC-3'; 18S forward 5'-GCCATATTTCCCATGAACG-3' and reverse 5'-GGCCTCATTAAACCATCCAA-3'.

Relative gene fold enrichments were determined by the 2−ΔΔCt method using the ribosomal unit 18S as a housekeeping gene. Experiments were performed in duplicate with three samples per experiment. The data represent the media value and the standard deviation of the two experiments combined. ANOVA tests were performed to analyse statistical significance.

Albumin secretion

Media samples corresponding to days 3, 5, 7 and 10 hepatocyte sandwich cultures were obtained and kept at −20°C for further analysis. Albumin synthesis was measured with a specific rat albumin ELISA kit (Bethyl Laboratories, Montgomery, TX, USA, E110-125). Briefly, 96-well plates were coated with a sheep anti-rat albumin antibody (1:1000 in a carbonate-bicarbonate buffer pH 9.6) for an hour. After that, the wells were washed three times with washing buffer (50 mM Tris, 0.14 M NaCl, 0.05% Tween-20 pH 8) and they were blocked for 30 min. with blocking buffer: sheep anti-rat albumin-HRP conjugated antibody (1:20,000 dilution, Bethyl Laboratories, A110-134P), goat anti-HNF4-α antibody (1:100 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA, sc-6556), goat anti-actin antibody (1:500 dilution, Santa Cruz Biotechnology, sc-1615). Each membrane was then washed three times with blocking buffer. A secondary antibody HRP conjugated was used for detection by chemiluminescence. Goat polyclonal antimouse IgG-HRP conjugated (1:1000 dilution, Santa Cruz Biotechnology, sc-2002) was used for detection. The membranes were incubated for 6 hrs with the secondary antibody, then washed once for 20 min. with blocking buffer and twice with TBS. Western blots were developed using a chemiluminescent system (Supersignal West Pico Chemiluminescent Substrate, Pierce Biotechnology, 34080) and protein bands visualized using an FluorChem gel imaging system (FluorChem, Alpha Innotech, St. Leandro, CA, USA).

Urea production

Media samples from hepatocyte sandwich cultures were collected from peptide and collagen cultures and stored at −20°C until analysis. Urea was quantitated using a urease method. Briefly, 10 µl of sample were incubated with 25 µl of urease solution (Sigma-Aldrich, St. Louis, MO, USA, US-3383) for 20 min. at room temperature. After that time, 50 µl of phenol nitroprusside (Sigma-Aldrich, P6994), 50 µl of alkaline hypochlorite (Sigma-Aldrich, A1727) and 200 µl of Milli-Q water were added to each well, and were incubated for 30 min. at room temperature. Absorbance was measured at 590 nm. Absorbance values were quantified using a calibration curve ranging from 0 to 150 mg urea/l, and values were normalized against the cell lysate total protein value. Each experiment was performed by duplicate with three samples per experiment. The data represent the media value and the standard deviation of the two experiments combined. ANOVA tests were performed to analyse statistical significance.

Total protein quantification

Hepatocytes were lysed using 300 µl of RIPA buffer (Boston Bioproducts, Worcester, MA, USA, BP-115D) containing protease inhibitors (Complete Mini protease inhibitor cocktail, Roche, 1836153). Lysates were sonicated for 5 min. and the solutions were centrifuged for 10 min. at 10,000 rpm to remove cell and scaffold debris. Total protein contained in the supernatant was quantified using the BCA assay kit (Pierce Biotechnology, Rockford, IL, USA, 23225) following the manufacturer's instructions.

SDS-electrophoresis and Western blotting

Hepatocytes for each culture condition were lysed using RIPA buffer (Boston Bioproducts, BP-115D) containing a cocktail of protease inhibitors (Roche Complete mini, Indianapolis, IN, USA, 1836153), samples were sonicated and maintained at −20°C until analysed. Total protein was quantitated with a BCA Protein Assay kit (Pierce Biotechnology, 33255). Volumes containing 5 µg of protein were mixed with sample buffer (Invitrogen, NP0007) boiled for 5 min. and loaded into a 12% Bis-Tris gel electrophoresis system (Invitrogen, NP0301). Proteins were electrophoresed using MOPS buffer (Invitrogen, NP0001), and then they were transferred for 2 hrs to a polyvinylidene difluoride membrane (Invitrogen, LC2002) and used to perform Western blot. The membranes were first blocked for 3 hrs with blocking buffer (5% dry skim milk in TBST buffer). Then they were incubated independently overnight at 4°C with several antibodies diluted in the same blocking buffer: sheep anti-rat albumin-HRP conjugated antibody (1:20,000 dilution, Bethyl Laboratories, A110-134P), goat anti-HNF4-α antibody (1:200 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA, sc-6556), goat anti-actin antibody (1:500 dilution, Santa Cruz Biotechnology, sc-1615). Each membrane was then washed three times with blocking buffer. A secondary antibody HRP conjugated was used for detection by chemiluminescence. Goat polyclonal antимouse IgG-HRP conjugated (1:1000 dilution, Santa Cruz Biotechnology, sc-2002) was used for detection. The membranes were incubated for 6 hrs with the secondary antibody, then washed once for 20 min. with blocking buffer and twice with TBS. Western blots were developed using a chemiluminescent system (Supersignal West Pico Chemiluminescent Substrate, Pierce Biotechnology, 34080) and protein bands visualized using an FluorChem gel imaging system (FluorChem, Alpha Innotech, St. Leandro, CA, USA).
For biotin dependent carboxylases detection, the membranes were blocked with blocking buffer for 3 hrs and then incubated overnight at 4°C with avidin-HRP conjugated in TBS (1:2000 dilution, Peprotech, Rocky Hill, NJ, USA). Membranes were washed t times with TBS and bands were visualized by chemiluminescence as explained above.

**Analysis of secreted protein by a proteomic approach**

Media samples from hepatocyte sandwich cultures were electrophoresed using a 12% Bis-Tris gels and MOPS buffer. Gels were stained with coomassie blue staining solution and HCM was loaded as a control. Differential bands from samples to controls were cut and identified using a proteomic approach. Briefly, bands were desalted and digested with trypsin, and eluted using an Agilent 1100 Nanoflow HPLC system connected to a Thermo Electron LTQ Ion Trap (Waltham, MA, USA) mass spectrometer. Ions were scanned in a range molecular weight range from 500 to 3500 Da. Probability-based validation of protein identifications were performed with a modified SEQUEST algorithm to match the fragmentation patterns with the theoretical fragment patterns of tryptic peptides in the Uniprot Rat protein database. Ions were considered valid when Xcorr was 2.0 for peptides with $m/z = 1$, 2.5 for peptides with $m/z = 2$ and 3.5 for peptides with $m/z = 3$.

**Cytochrome CYP3A2 induction with dexamethasone**

Cells (65,000 cells/cm²) were cultured for 5 days under standard conditions described above and medium containing 10 μM dexamethasone (DEX; MP Biomedicals, Solon, OH, USA, 190040) was added on day 6. RNA samples were taken at day 7 for analysis of the cytochrome CYP3A2 by qPCR.

**Results**

The use of peptide scaffolds for long-term hepatic cultures were evaluated by determining liver-specific functions of hepatocytes cultured in such systems. The results obtained were compared with those from the standard collagen sandwich model used as a control. The collagen and peptide hepatocyte sandwich cultures were prepared as shown in Fig. 1. Matrix substrates were prepared first, then hepatocytes were added, incubated overnight at 37°C, washed to remove non-adherent cells and finally another layer of gel was added to entrap the cells in a sandwich fashion. For the peptide hydrogel matrices, a 0.5% solution in sucrose 10% was gelled through contact with culture medium (see ‘Materials and methods’).

We first observed that the morphology and appearance of hepatocytes cultured in both collagen and peptide hydrogel sandwich systems were similar over the entire culture period (Fig. 2A). In all cases – including cultures with functionalized peptide hydrogels – hepatocytes became flattened, adopted an in vivo-like polygonal shape and by day 3 established extensive cell–cell contacts with a reorganization of bile canaliculi-like structures. To demonstrate the functionality of the intercellular biliary zones, the cultures were incubated in presence of FDA. We observed that they were capable of metabolizing and transporting the dye from the cytoplasm into the canaliculi-like structures, as demonstrated by fluorescence microscopy (Fig. 2B). A similar observation has (previously) been reported for primary hepatocytes in collagen sandwich cultures [24–26].

Moreover, we wanted to assess gene expression analysis by means of quantitative PCR between freshly isolated hepatocytes and our culture systems (Fig. 3). In addition, because matrix thickness might influence nutrient and oxygen transport, and therefore hepatocyte function, we studied gene expression in cultures with 1 mm and 0.5 mm gel heights [27] (see ‘Materials and methods’).

As metrics of hepatocyte differentiation, we examined albumin and
HNF4-α, one hepatic metabolic enzyme, tyrosine aminotransferase (TAT) and MDR2 as a canalicular membrane marker transporter [28] on day 7. We observed that compared with the 1 mm hydrogel cultures, the use of the 0.5 mm collagen and peptide matrices resulted in a higher expression of all the genes studied, suggesting that mass transfer through the hydrogel is affected by the gel thickness (Fig. 3). Therefore, we decided to use the 0.5-mm-thick gels for the rest of experiments and we included the modified self-assembling peptide TAG ('Materials and methods').

Despite the down-regulation of most of the genes studied in the 1 mm gels relative to freshly isolated hepatocytes (Fig. 3A), it can be observed that the gene expression profile was similar in the collagen and the peptide sandwiches. The functionalized peptide YIG-containing matrix exhibited the highest levels of gene expression for all genes studied (Fig. 3A). Nevertheless, the gene expression for MDR2 remained strongly down-regulated as compared to freshly isolated hepatocytes over the entire culture period (Fig. 3A). Looking deeper into the gene expression analysis for the 0.5 mm gels, it can be observed that in general peptide hydrogel and collagen cultures remained mainly in the same levels of expression. Relative expression values that fell between −1 and 1 were considered the same as fresh hepatocytes. In all conditions tested it was observed that albumin and HNF4-α have already reached the expression levels of freshly isolated hepatocytes. In the case of albumin, all the peptide hydrogel cultures were significantly different than collagen. For HNF4-α significant differences were only observed for RAD16-I and YIG. In addition, MDR2 remained down-regulated in almost all conditions tested, except for YIG, which reached the relative levels of freshly isolated hepatocytes, and significantly different than collagen (Fig. 3B). However, RAD16-I and TAG presented a slight but significative up-regulation in MDR2 compared to collagen (Fig. 3B). Moreover, TAT in collagen cultures remained somehow down-regulated, but its expression in peptide hydrogel sandwich cultures was comparable to the positive control (freshly isolated hepatocytes), and significantly different than collagen cultures (Fig. 3B).
The effect of peptide hydrogels on hepatocyte-specific functionality was evaluated and compared with the collagen sandwich system by assessing cell-specific albumin and urea secretion over the entire culture period. In all culture systems, the albumin secretory capacity gradually increased between culture days 3 and 7 and remained stable thereafter (Fig. 4A). As shown by immunoblotting, albumin was expressed at a level that was similar in all hepatocyte cultures on days 7 and (Fig. 4B and C).

Furthermore, urea present in the media (indicative of amino acid catabolism) was analysed (Fig. 5). Under all culture conditions, the urea concentration slightly decreased between days 3 and 5 of culture and did not change thereafter (Fig. 5A). Similar to albumin, levels of urea production were comparable in the collagen sandwich and peptide scaffold cultures (Fig. 5A). Western blots analysis of the transcription factor HNF4-α demonstrated that it is expressed in all culture conditions (Fig. 5B). However, compared to freshly isolated hepatocytes control, an additional band of higher molecular weight was detected in all culture conditions, suggesting that the in vitro system might be synthesizing an additional isoform also recognized by the antibody against-HNF4-α. Additionally, we detected biotin-dependent carboxylases, which are enzymes highly expressed in liver [29–31]. In mammals, only four different biotin-dependent carboxylases have been detected so far, acetyl-CoA carboxylase (ACC, MW 270 kD), pyruvate carboxylase (PC, MW 125 kD), 3-methylcrotonyl-CoA carboxylase (MCC, MW 75 kD) and propionyl-CoA carboxylase (PPC, MW 72 kD) [29–31]. These enzymes, are involved in the oxidation of odd-chain fatty acids, catabolism of branched amino acids such as leucine, lipogenesis and gluconeogenesis, respectively [29–31]. Using an assay, based on biotin-avidin interaction, we were able to detect three biotin-dependent carboxylases also present in freshly isolated hepatocytes (Fig. 5C). These enzymes were also present in all peptide hydrogel cultures, but interestingly, collagen sandwich cultures did not appear to express PPC (Fig. 5C).
**Fig. 4** Analysis of secreted albumin. 
(A) Analysis of secreted rat albumin present in the media normalized by total protein present in the cellular lysate. Data are presented as mean ± S.D. from two different experiments. 
COLL: collagen. (B and C) Albumin Western blot of the cellular lysate. F.H: Freshly isolated hepatocytes.

**Fig. 5** Analysis of adult hepatocyte markers. 
(A) Urea present in the media at different time-points. The results are normalized against total cellular protein present in the lysates. Data are presented as mean ± S.D. from two different experiments. COLL: collagen (B) HNF4-α Western blots (C) Biotin dependent carboxylases present in the protein lysates. PC: pyruvate carboxylase, MCC: methylcrotonyl-CoA carboxylase and PCC: propionyl-CoA carboxylase. F.H.: Freshly isolated hepatocytes.
Moreover, we examined and analysed the secreted proteome of hepatocytes in culture. This is a very interesting approach, since hepatocytes secrete a large amount of plasma proteins and some of them can be used as markers for diagnosing diseases [32]. Therefore, we ran coomassie blue-stained protein gels of culture media obtained at day 7 and day 10 for collagen and RAD16-I peptide hydrogel culture systems (Fig. 6). We included media as our control in one lane in order to differentiate medium containing proteins from the ones secreted into the medium. We analysed bands present in both collagen and RAD16-I ensuring that the isolated material is released by the hepatocytes. Four differential bands for both collagen and RAD16-I were identified and were then excised from the gels. The proteins contained in each band were tryptic digested and the remaining peptides were separated and analysed by nanoflow-HPLC MS/MS (‘Materials and methods’). A brief report of the proteins obtained and their function is showed in Table 1. Several proteins were identified in both matrix systems (collagen and RAD16-I) Table 1. They are mainly plasma proteins or protease inhibitors, and their function is briefly described in Table 1 [33–49]. These findings are in concordance with a previous study using rat hepatocyte collagen sandwich cultures [32]. In some other cases, protein function is still unknown such as hypothetical protein Q4FZU2. Also, other proteins such as α1-macroglobulin were found in a zone of lower molecular weight than expected, which could be due to proteolysis during the culture period (Table 1).

Inducibility of CYP3A2 by DEX using rat hepatic culture models has previously been described [6, 50, 51]. Thus, it was of interest to investigate whether hepatocytes also respond to DEX by overexpressing CYP3A2 when cultured in the peptide scaffolds. The CYP3A2 gene exhibited a high down-regulation relative to freshly isolated hepatocytes in peptide and collagen sandwich configuration at day 7 (Fig. 7A). However, when cultures were incubated for 24 hrs with DEX 10 μM the expression was up-regulated in both collagen and peptide sandwiches compared to untreated cells, indicating that hepatocyte in sandwiched cultures have the ability to respond to external inductive stimuli (Fig. 7B).

**Discussion**

Tissue architecture and parenchymal cell morphology reflect the functional differentiation of the liver. Hepatocytes are arranged as monolayer plates in vivo, an organization which allows the cells to be highly vascularized. Sandwiching hepatocytes between two layers of collagen has shown to provide the cells with an in vivo-like environment that enables the cells to preserve their specific functionality for several weeks [3–5]. In this work we have used synthetic extracellular matrix analogues in order to recreate the most commonly used hepatocyte culture system in vitro, the collagen sandwich [3–5]. We suggest that the geometrical factor in combination with the use of an artificial extracellular matrix may provide the hepatocytes a unique microenvironment which might allow them to retain important liver-specific functions. In this study, all experiments were performed in parallel to collagen sandwich system, which is considered today as the gold standard for the culture of hepatocytes in vitro [3–5].

We have observed that in peptide cultures hepatocyte morphology appears to be polygonal and with a vast network of bile canalicular-like structures as it happens in collagen cultures (Fig. 2A and B). These canalicular-like structures proved to be functional by the fact that they can metabolize and transport FDA through them (Fig. 2B), as it had been previously shown for collagen sandwich cultures [24, 25]. We chose some hepatocyte markers to assess function and gene and protein expression. In terms of gene expression, the levels of relative expression of the genes studied was comparable, except for hepatocytes cultured in peptide hydrogel sandwiches containing the peptide YIG, which presented some degree of up-regulation especially for HNF4α and albumin. Generally speaking, for all the functional experiments tested, hepatocytes in peptide sandwich cultures behaved correspondingly similar to collagen sandwich cultures. The same happened in terms of gene and protein expression, and in relation to the proteomic profile secreted to the culture medium. However, further efforts are required to improve the system to fully express liver-specific genes and to perform liver-specific functions. For
Fig. 7 DEX induction of CYP3A2 expression. (A) CYP3A2 gene expression relative to freshly isolated hepatocytes by quantitative PCR at different time-points. (B) CYP3A2 expression at day 7, after a 24 hrs induction with DEX 10 µM, COLL: collagen. Data were obtained from two independent experiments, each of which was performed normalizing to the 18s corresponding to each hepatocyte isolation. Data are presented as mean ± S.D. (*P < 0.001, **P < 0.01, ***P < 0.05).

Table 1 Secreted proteins found in both collagen and RAD16-I culture medium

| Protein                        | Band | MW  | Function                                             | References |
|--------------------------------|------|-----|------------------------------------------------------|------------|
| Apolipoprotein A1 (ApoA1)      | 1    | 30 kD | Involved in reverse cholesterol transport           | [33–35]    |
| α1-microglobulin/bikunin precursor (AMBP) | 1    | 39 kD | a-1-microglobulin has immunoregulatory properties. Binds to IgGA Bikunin is a serine protease inhibitor | [36–38]    |
| Apolipoprotein E (ApoE)        | 2    | 36 kD | Lipid transport. Formation of VLDL and chylomicrons | [39]       |
| Haptoglobin                    | 2    | 38.5 kD | Free hemoglobin turnover                          | [40, 41]  |
| Apolipoprotein J (ApoJ, clusterin) | 2    | 51 kD | Immune regulation, lipid transport, remodeling, apoptosis | [42–44]    |
| Q4FZU2                         | 2    | 59 kD | Hypothetical protein. Unknown                       |           |
| α1-macroglubulin*              | 2    | 167 kD | Protease inhibitor                                   | [45]       |
| Retinol binding protein RETBP  | 3    | 23 kD | Transports retinol from liver                       | [46]       |
| Fibronectin                    | 4    | 272 kD | Extracellular matrix and plasma protein             | [47]       |
| α1 inhibitor III               | 4    | 165 kD | Protease inhibitor                                   | [48, 49]  |

*Indicates proteins detected in a MW zone where they were not expected to be found.
example, it would be essential for the implementation of the system in drug screening applications, the expression of cytochromes P450 or phase I enzymes which are the responsible of the biotransformation of drugs and other xenobiotics. In particular, CYP3A2, which represents the 30% of the total cytochromes P450, metabolizes the 50% of the marketed drugs. CYP P450, especially CYP3A2, is lost rapidly after hepatocyte isolation and its expression is recovered with difficulty. However, it can be induced chemically with DEX. In collagen and peptide hydrogel sandwich systems, CYP3A2 appears to be highly down-regulated (Fig 7A), but it is induced significantly in both systems using DEX (Fig. 7B). To overcome this drawback, different strategies can be used to better recreate the hepatocyte microenvironment, such as the use of a hepatocyte non-parenchymal cell coculture or the integration of the hydrogel sandwich into a bioreactor that will allow better oxygen and mass transfer, as previously described for other systems [13–17]. Nevertheless, this peptide sandwich model represents an enormous advance through the use of a defined and synthetic extracellular matrices analogue to obtain a truly comparable system to what is considered the gold standard in long-term hepatocyte in vitro culture. We suggest that these promising results are an important beginning for the application of the self-assembling peptide hydrogel in hepatic reconstruction in vitro for many biomedical applications.

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