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3D hepatocyte monolayer on hybrid RGD/galactose substratum

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Abstract

Hepatocyte-based applications such as xenobiotics metabolism and toxicity studies usually require hepatocytes anchoring onto flat substrata that support their functional maintenance. Conventional cell culture plates coated with natural matrices or synthetic ligands allow hepatocytes to adhere tightly as two-dimensional (2D) monolayer but these tightly anchored hepatocytes rapidly lose their differentiated functions. On galactosylated substrata, hepatocytes adhere loosely; and readily form three-dimensional (3D) spheroids that can maintain high levels of cellular functions. These spheroids detach easily from the substrata and exhibit poor mass transport properties unsuitable for many applications. Here, we have developed a hybrid RGD/galactose substratum based on polyethylene terephthalate film conjugated with both RGD peptide and galactose ligand to enhance cell adhesion and functions synergistically. Primary hepatocytes adhere effectively onto the transparent hybrid substratum in 96-well plates as monolayer while exhibiting high levels of liver-specific functions, morphology and cell–cell interactions typically seen in the 3D hepatocyte spheroids. The hepatocytes cultured onto the hybrid substratum also exhibit high levels of sensitivity to a model drug acetaminophen similar to the 3D hepatocyte spheroids. The monolayer of hepatocytes exhibiting the 3D cell behaviors on this flat hybrid substratum can be useful for various applications requiring both effective mass transfer and cellular support.
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1. Introduction

Primary hepatocytes have been widely used in bioartificial liver-assisted devices (BLAD), pharmacological, toxicological and metabolic studies. Hepatocytes in these applications are typically cultured on appropriate substrata to achieve optimal cell attachment and functional maintenance [1–3]. A variety of natural or synthetic polymeric substrata have been employed for hepatocytes culture (e.g. plastic surfaces or membranes coated with extracellular matrix proteins such as collagen, laminin, fibronectin or conjugated with cell adhesion peptides, such as Arg-Gly-Asp (RGD) [4] and Tyr-Ile-Gly-Ser-Arg (YIGSR) [5]). Hepatocytes anchor tightly to these substrata, and exhibit extended and spreading cell morphology, with low levels of liver-specific functions likely due to hepatocyte de-differentiation [6]. These substrata have been extensively used for xenobiotics screening in microplates [7–9] as well as for BLAD bioreactors [10].

Galactose conjugated substrata are attractive alternatives for hepatocyte attachment through the galactose-asialoglycoprotein receptor (ASGPR) interaction [11]. Several strategies have been adopted to fabricate substrata conjugated with galactose ligand [12]. Hepatocytes cultured on these galactosylated substrata tend to maintain round cell morphology; and self-assemble into three-dimensional

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(3D) spheroids in the presence of epidermal growth factor (EGF) [12]. Hepatocyte spheroids are 3D compact multicellular spherical aggregates which are believed to recreate an *in vivo*-like microenvironment with abundant cytoplasmic organelles and the presence of bile canaliculi, tight junctions and gap junctions [13,14]. Spheroids are also reported to maintain several liver-specific functions such as albumin secretion and cytochrome P450 detoxicification activity in culture for weeks. However, the usefulness of 3D hepatocyte spheroids in applications is limited due to the poor mass transport of nutrients, oxygen, xenobiotics and metabolites into and from the core of these large cellular aggregates [14,15]. Cell loss is also a critical issue in forming and maintaining these spheroids in applications since the spheroids detach easily from the substratum [16,17].

We have developed here a bioactive substratum by conjugating both RGD peptide and galactose ligand to polyethylene terephthalate (PET) film such that hepatocytes could anchor stably onto the substratum as monolayer while maintaining limited-spreading cell morphology, tight cell–cell interactions and high levels of liver-specific functions typically seen in 3D hepatocyte spheroids. We named this hepatocyte culture configuration as 3D hepatocyte monolayer.

PET film was surface-modified with acrylic acid (AAc) followed by ligands conjugation in a 'two-step' 'EDC' chemistry. The PET film conjugated with both the RGD peptide and galactose ligand (PET-Hybrid) was inserted into 96-well microplates for hepatocyte culture, with PET film conjugated with only RGD peptide (PET-RGD), galactosylated PET (PET-Gal) and collagen-coated microplates as controls. Analysis of the distribution of key markers (F-actin, E-cadherin, p-focal adhesion kinase (FAK) and liver-specific functions (albumin and urea synthesis, P450 detoxicification activity) indicated that the cells on PET-Hybrid film behaved like cells in 3D spheroids even though they adhered well to the two-dimensional (2D) substratum. The 3D hepatocyte monolayer cultured on PET-Hybrid also exhibited similar sensitivity to acetaminophen (APAP)-induced hepatocytotoxicity as the 3D hepatocyte spheroids rather than the 2D monolayer control. This hybrid RGD/galactose bioactive substratum supports spheroid-like hepatocyte behaviors on monolayer, avoiding the cell loss and mass transfer limitation of typical 3D spheroids, and can be immediately adopted in microplates or other prevailing 2D culture devices or bioreactors for various applications.

2. Materials and Methods

2.1. Materials

Biaxially oriented PET films of about 100 μm in thickness were purchased from Goodfellow Inc. of Cambridge, UK. The galactose ligand, 1-*O*-(6'-aminohexyl)-D-galactopyranoside (AHG, M.W. 279) was synthesized according to the method developed previously [6,18,19] and verified by NMR spectrum. RGD peptide (GRGDS) was purchased from Peptides International. All other chemicals were purchased from Sigma–Aldrich, Singapore unless otherwise stated.

2.2. Fabricating PET film grafted with poly acrylic acid (PET-pAAc)

Poly-acrylic acid (pAAc) was grafted onto the PET film surface with a modified protocol [6,20] for conjugating bioactive ligands. Briefly, PET film was cut into 2 cm × 8 cm strips and cleaned in ethanol. The air-dried PET strips were subject to argon plasma treatment which was carried out in SAMCO Basic Plasma Kit (SAMCO International Inc.) operating at a radio frequency (RF) of 13.6 MHz. Argon was introduced into the chamber in the SAMCO kit at a flow rate of 50 ml/min with chamber pressure maintained at 20 Pa. Plasma was generated at an electric power of 40 W for 1 min. After the plasma treatment, the PET strips were exposed to atmosphere for 10 min to promote the formation of surface peroxides and hydroperoxides, which were used for the subsequent UV-induced grafting of pAAc. For the UV-treatment, quartz tubes with length of 12 cm and diameter of 2.5 cm were fabricated at the Glassware workshop of the Department of Chemistry at NUS. The plasma-treated PET-strip was immersed in 30 ml of the aqueous solution containing AAc in the quartz tube. Argon was bubbled through the solution to thoroughly remove oxygen. The quartz tube was capped tightly and placed in water bath with constant temperature of 28 °C and subjected to UV irradiation for 30 min using a 400 W flood lamp in UV-F 400 unit (Panacol-Elosol GmbH). After grafting, the PET strip was taken out of the tube and washed extensively with deionized water for 24 h to remove the residual homopolymer absorbed on the surface.

2.3. Fabricating bioactive substrata by conjugating RGD and/or Gal ligands onto PET-pAAc film via 'two-step' EDC chemistry Fig. 1

PET-pAAc strips were cut into circular disks with diameter of 6.4 mm in order to fit into the 96-well microplates. RGD peptide and galactose ligand were conjugated via amide bonds onto PET-pAAc separately or simultaneously using a 'two steps' EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride) chemistry (Fig. 1). Briefly, at the first step, 100 μl of MES buffer (50 mM, pH of 5.5) containing 1.5 mg EDC and 0.3 mg sulfo-NHS were added to each 96-well containing the PET-pAAc disk to activate the surface carboxylic groups by forming NHS esters. After 2 h activation at room temperature, the MES solution was completely removed and replenished with 100 μl phosphate buffer (0.1 M, pH of 7.4) containing ligands and allowed to react with activated substratum by shaking at 300 rpm in a thermomixer (Eppendorf) for 48 h at 4 °C. PET-RGD or PET-Gal was fabricated by reaction with RGD peptide or galactose ligand, respectively. PET-Hybrid was fabricated by reaction with homogeneous mixture of RGD peptide and galactose ligand with different ratios. After conjugation of the bioactive ligands, each sample was quenched with 0.5% ethanolamine solution for 15 min to block non-specific interactions of the un-reacted carboxylic groups with the hepatocytes. The microplates containing different substrata were sterilized by soaking in 70% ethanol for 3 h and then rinsed 3 times with PBS. Collagen substratum was prepared by incubating 100 μl of 1.5 mg/ml collagen solution into each well of the 96-well microplates overnight at 4 °C. The excess collagen solution was aspirated and each well rinsed 3 times with PBS.

2.4. Characterization of PET-RGD, PET-Gal and PET-Hybrid substrata

2.4.1. Determination of the surface-grafted pAAc concentration

The density of the graft carboxylic groups on the PET films was determined by a colorimetric method using Toluidine Blue O staining previously reported [6,21].

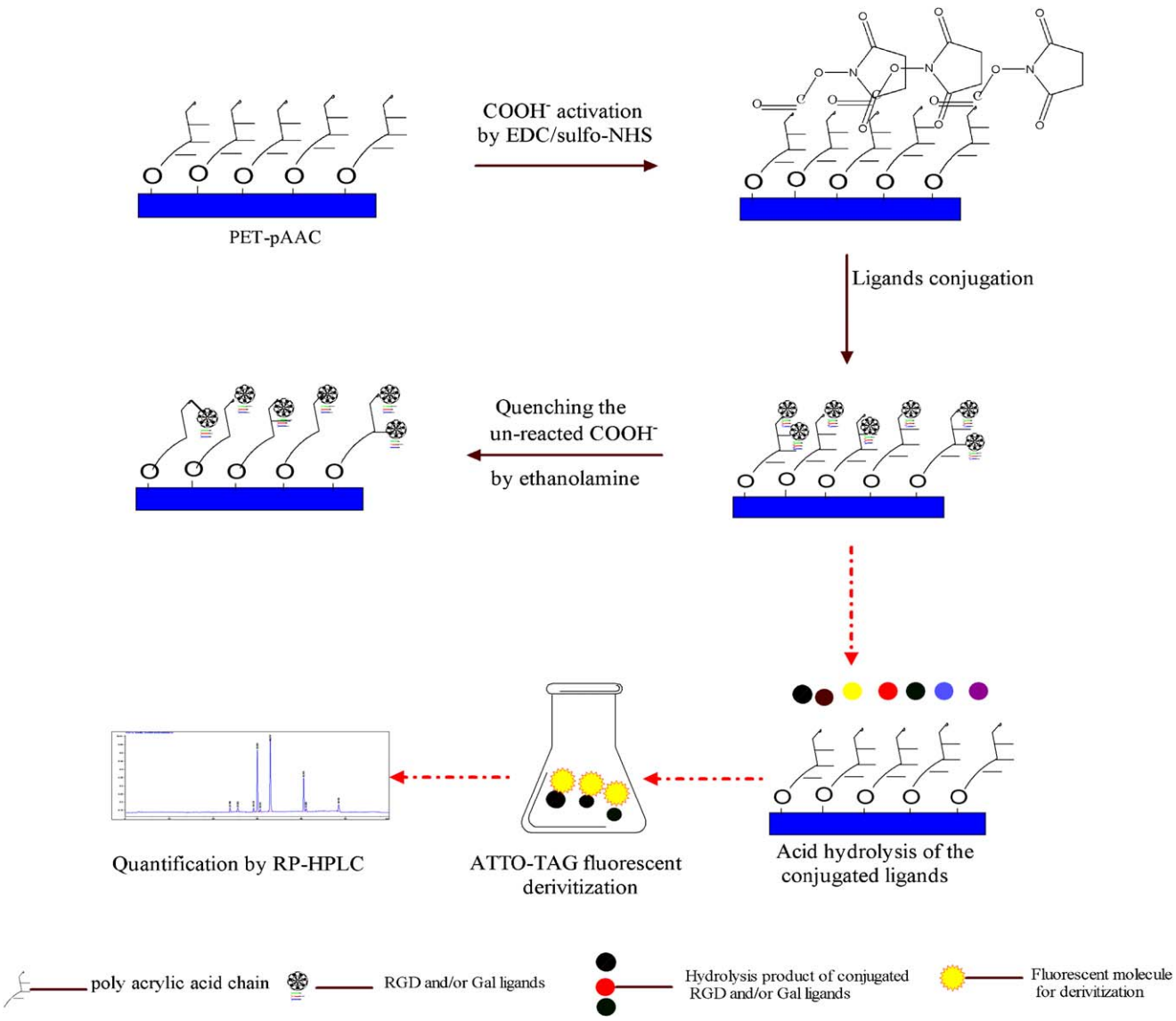

Fig. 1. Schematic diagram of ligands conjugation onto PET-pAAc by a 2-step reaction scheme (solid arrows) and quantitative analysis of the conjugated ligands by RP-HPLC (dotted arrows).

2.4.2. X-ray photoelectron spectroscopy (XPS) measurements

XPS was used to qualitatively verify the pAAc grafting and ligand conjugation onto the PET film. Measurements were made on a VG ESCALAB Mk II spectrometer with MgKα X-ray source (1253.6 eV photons) at a constant retard ratio of 40.

2.4.3. RP-HPLC quantification of the conjugated RGD peptide and Gal ligand on PET

RGD peptide and/or Gal ligand on PET were hydrolyzed off the substrata using Acid Hydrolysis Station (C.A.T. GmbH & Co.) in 6 N HCl at 110 °C for 24 h under vacuum. The cooled hydrolyzed solution was filtered into a new vial and evaporated under nitrogen. The hydrolyzed ligands from PET were re-suspended in 50 µl DI-water and derivatized using ATTO-TAG™ CBQCA Amine-Derivatization Kit (Molecular Probes) for fluorescence detection after separation on a reverse phase C-18 column in HPLC (Agilent Technology). Optimized operational conditions: mobile phase: A: water + 0.1% TFA, B: acetonitrile + 0.1% TFA; gradient: A/B (98:2) to (70:30) in 45 min; flow rate: 1 ml/min; fluorescence detection: excitation@450 nm, emission@550 nm. Standard curves were established against hydrolysis product of soluble RGD peptide and Gal ligand, respectively. Among the hydrolysis product of RGD peptide, the peak corresponding to arginine was chosen to represent and quantify RGD peptide due to its sharpness and early elution time in the chromatograph.

2.5. Hepatocyte isolation and culture

Hepatocytes were harvested from male Wistar rats weighing 250–300 g by a two-step in situ collagenase perfusion method [22]. Animals were handled according to the IACUC protocol approved by the IACUC committee of the National University of Singapore. Viability of the hepatocytes was determined to be >90% by Trypan Blue exclusion assay and a yield of $>10^8$ cells/rat.

Freshly isolated rat hepatocytes (3.2×10^4) were seeded onto different substrata at 1×10^5 cells/cm^2 within 96-well microplate and cultured in 100 µl of William's E culture medium supplemented with 10 mM $NaHCO_3$, 1 mg/ml BSA, 10 ng/ml of EGF, 0.5 µg/ml of insulin, 5 nM dexamethasone, 50 ng/ml linoleic acid, 100 units/ml penicillin, and 100 µg/ml streptomycin. Cells were incubated with 5% CO_2 at 37°C and 95% humidity. After 2 h

incubation, culture medium containing the unattached cells was removed; the wells were rinsed with PBS and replenished with fresh culture medium daily for 7 days.

Hepatocyte attachment on different substrata after 2 h was calculated based on a DNA analysis method [23]. Attached cells were lysed on the substrata by a freeze-thaw cycle of freezing in DNA-free DI-water at −80 °C overnight and thawing at 37 °C. DNA concentration was determined using PicoGreen® dsDNA quantitation kit (Molecular Probe). The attached cell number was determined using a standard curve generated from the DNA concentrations of known number of cells. Hepatocyte attachment on different substrata was expressed as the seeding efficiency (attached cell number divided by total cell number initially seeded).

2.6. Immuno-fluorescence microscopy

For F-actin, phosphorylated-FAK (p-FAK), and E-Cadherin staining, hepatocytes cultured for 3 days on different substrata were fixed in 3.7% para-formaldehyde (PFA). For staining F-actin, the cells were permeabilized for 5 min in 0.1% Trion X-100 plus 1% bovine serum albumin (BSA) and incubated with TRITC-phalloidin (1 μg/ml) for 20 min. For staining p-FAK, the permeabilized cells were blocked with 1% BSA for 1 h and incubated for 1 h in 25 μg/ml of polyclonal antibody of p-FAK (Upstate) and subsequently incubated for 1 h with TRITC-conjugated goat-anti-rabbit secondary antibody. For staining E-cadherin, fixed cells were blocked with 1% BSA for 1 h and incubated with 10 μg/ml E-cadherin monoclonal antibody for 3 h followed by incubation for 1 h with TRITC-conjugated rabbit anti-mouse secondary antibody. 3D stack of confocal microscopy images were acquired with 63× NA1.4 oil lens on a Zeiss Meta 510 Confocal Microscope. The image stack was reconstructed in a 3D projection with maximum intensity projection algorithm (Zeiss LSM 510).

2.7. Hepatocyte functions

Total DNA content per sample measured by the PicoGreen DNA assay was used to normalize the function data to account for the cell loss from different substrata throughout the 7-day culture [24,25].

2.7.1. Albumin secretion and urea synthesis

The albumin secretion by hepatocytes on days 1, 3, 5 and 7 were measured using the Rat Albumin ELISA Quantitation Kit (Bethyl Laboratories Inc., Montgomery, Texas). Urea synthesis by hepatocytes cultured in phenol-red-free William's E culture medium with 2 mM NH_4Cl for 90 min were measured on days 1, 3, 5 and 7 using the Urea Nitrogen Kit (Stanbio Laboratory, Boerne, Texas).

2.7.2. Induction and measurement of cytochrome P-450 1A activity

Cytochrome P-450 1A1/2 activity was induced by adding 3-methylcholanthrene (3MC) and measured by 7-ethoxyresorufin-*O*-deethylation (EROD) assay. Briefly, 2 μM of 3MC was added to the hepatocyte culture medium 24 h before measuring the EROD activity. On days 1, 3, 5 and 7, hepatocytes induced by 3MC were incubated with phenol-red-free culture medium containing 8 μM 7-ethoxyresorufin (7ER) substrate and 80 μM dicumarol. Dicumarol was added to prevent degradation of the fluorescent resorufin product by cystolic oxidoreductases. After 1 h incubation, the 7ER-containing medium was collected and measured at 530 nm excitation/585 nm emission against resorufin standards using the microplate reader (Tecan Safire^2).

2.8. Acetaminophen-induced hepatotoxicity to hepatocytes

The hepatotoxicity testing of APAP was modified from procedures previously reported [26,27].

2.8.1. Preparation of toxin and exposure to hepatocytes

APAP was dissolved in DMSO and the final concentration of DMSO in the medium was kept at less than 0.2%. After pre-incubation for 24 h, hepatocytes cultured on PET-Gal, PET-Hybrid and collagen substratum in 96-well microplates were exposed to APAP (100 μl/well) with high (10 mM) and low concentration (2 mM), respectively, for 24 h or 48 h until cell viability was measured. 2 μM of 3MC was co-administered with APAP in the drug–drug interaction study.

2.8.2. Detection of APAP-induced hepatotoxicity

MTS assay using the CellTiter 96 Aqueous One Solution Reagent (Promega) was conducted to quantify cell viability. After treatment with test drugs, cells were exposed to 100 μl/well of 5× diluted MTS reagent in phenol-red-free William's E culture medium and incubated for 3 h at 37 °C. The absorption of MTS was measured at 450 nm using microplate reader (Tecan Safire^2). The response to the test drugs from the hepatocytes cultured on different substrata was expressed as the 'survival ratio', which was calculated by the MTS assay reading on hepatocytes exposed to drugs normalized to the MTS assay reading on drug-free control.

3. Results

3.1. Fabrication and characterization of PET film grafted with acrylic acid

pAAc was grafted onto the PET film by argon-plasma treatment and UV-induced copolymerization. The effectiveness of the grafting was demonstrated by XPS analysis (Fig. 2). The XPS wide scan spectrum of the pristine PET film showed peaks corresponding to C 1s (binding energy, 285 eV) and O 1s (binding energy, 532 eV), which revealed the presence of carbon and oxygen signals. The spectrum of PET-pAAc film (Fig. 2B) showed the same peaks as pristine PET film; however, the relative intensity ratio of oxygen to carbon peaks was higher in PET-pAAc film than in pristine PET film. The pAAc grafting density was quantified by TBO colorimetric assay [6,21]. PET-pAAc substrata with carboxyl group densities from 8.2 ± 2.3 to 258.2 ± 24.2 nmol/cm^2 could be obtained by varying the initial concentration of the AAc monomer solution from 1% to 5%. As reported previously, the difference in density of carboxylic groups and conjugated galactose ligand was not expected to lead to significant differences in 3D hepatocyte spheroid formation and functional maintenance when the densities were above certain value [6]. 3.75% AAc monomer solution was chosen to fabricate PET-pAAc with carboxyl-group density of 78.5 ± 10.2 nmol/cm^2 for the following ligands conjugation and cell culture in order to achieve the desirable ligand-conjugation density using the relatively inefficient two-step 'EDC chemistry' [28].

3.2. Fabrication and characterization of bioactive substrata

RGD peptide (GRGDS) and/or galactose ligand (AHG) were conjugated onto PET-pAAc (Fig. 1), and successful conjugation of ligands was confirmed by XPS (Fig. 2). In contrast to pristine PET and PET-pAAc, a new peak corresponding to N 1s (binding energy, 400 eV) introduced by bioactive ligands appeared in the spectra of PET-RGD, PET-Gal and PET-Hybrid. To measure the amount of the

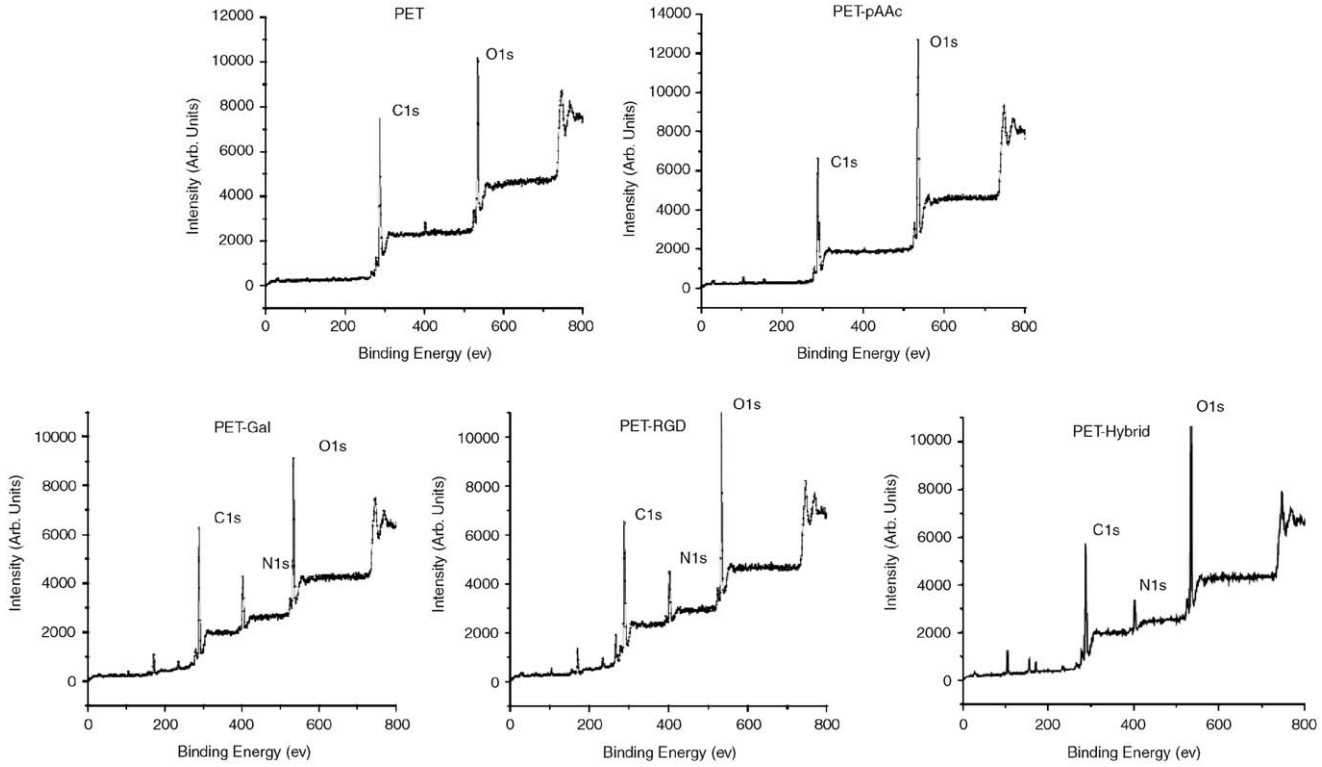

Fig. 2. XPS wide scanning spectrums of PET, PET-pAAc, PET-Gal, PET-RGD and PET-Hybrid which showed the successful grafting of polyacrylic acids and following conjugation of RGD peptide and galactose ligand onto PET-pAAc.

GRGDS and/or AHG conjugated onto the films, we removed the conjugated GRGDS and/or AHG from the film by acid hydrolysis; and quantified the hydrolyzed GRGDS and AHG by RP-HPLC with fluorescence detector after derivatizing the hydrolysis products of GRGDS and AHG to fluorescent substances. Representative chromatograms of different samples were illustrated in Fig. 3A. We controlled the ratio of the GRGDS and AHG in PET-Hybrid by monitoring the conjugation efficiencies of the GRGDS and AHG onto PET-pAAc, respectively. GRGDS exhibited higher conjugation efficiency than AHG (Fig. 3B, 3C). In total, 3 mg/ml (0.3 mg per 96 well) AHG and 0.6 mg/ml (0.06 mg per 96 well) GRGDS were chosen to react with the activated PET-pAAc to achieve a conjugation ratio of ~1:1. The final density of the conjugated GRGDS and AHG on the film was 5.63 ± 0.86 and 6.94 ± 0.74 nmol/cm^2, respectively. For 78.5 ± 10.2 nmol/cm^2 of carboxylic groups available on PET-pAAc, ~16% were conjugated with the ligands. To achieve 1:5 and 5:1 ratios of conjugated GRGDS and AHG, we used 0.12 and 3 mg/ml GRGDS, respectively, to co-conjugate with 3 mg/ml AHG onto PET-pAAc. The final densities of GRGDS/AHG were $1.31 \pm 0.49/5.38 \pm 0.89$ and $19.40 \pm 3.19/4.36 \pm 0.45$ nmol/cm^2. For PET-Gal or PET-RGD, 3 mg/ml AHG or 0.6 mg/ml GRGDS was reacted with the activated PET-pAAc, respectively. The density of conjugated AHG of PET-Gal was 5.92 ± 0.74 nmol/cm^2 and the density of conjugated GRGDS of PET-RGD was 7.04 ± 0.96 nmol/cm^2.

3.3. Hepatocyte attachment on bioactive substrata over time

Ligands conjugation significantly enhanced hepatocyte attachment onto the bioactive substrata after 2 h of seeding (Fig. 4A). Hepatocytes attached very well to PET-Gal, PET-Hybrid and collagen substratum; and attached less well to PET-RGD, and poorly to PET-pAAc and tissue culture plate (TCP). Similar numbers of hepatocytes attached to PET-Hybrid with RGD:galactose ratios of 1:5, 5:1 or 1:1 (data not shown).

Cultured hepatocytes showed different attachment on various substrata during the 7-day culture as shown by the total DNA content change (Fig. 4B). Total DNA content was used as an estimate of viable cells attached to the substratum [24,29]. The gradual decrease of the total DNA content was likely the result of the cell loss during the daily medium change. As the 3D spheroids began to detach from the substratum after day 3, a rapid drop in DNA content of hepatocytes cultured on PET-Gal was observed. On the other substrata, a slower decrease in DNA content was observed. The low total DNA content of hepatocytes cultured on PET-RGD was likely due to the lower cell number initially seeded. Hepatocytes cultured on collagen substratum and PET-Hybrid showed significantly better attachment than on PET-Gal from day 5 onwards ($P < 0.01$).

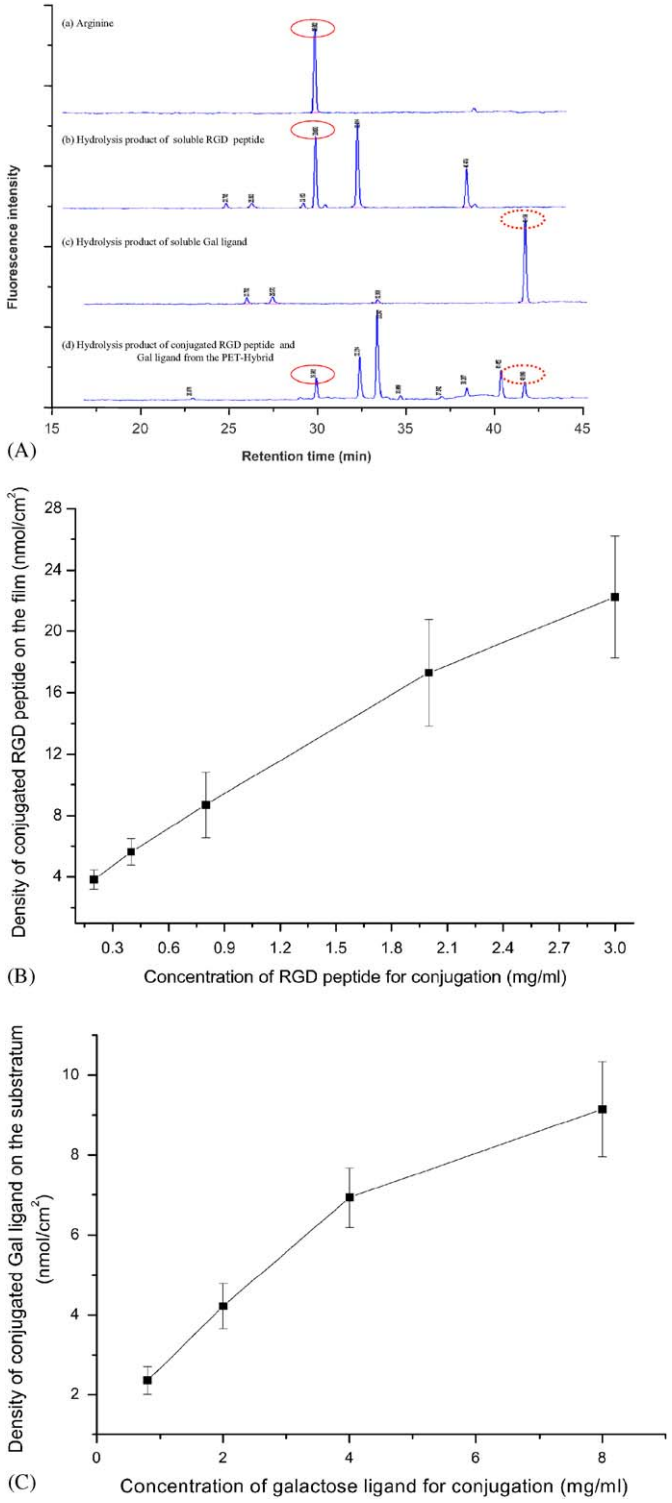

Fig. 3. Quantitative analysis of the conjugated RGD peptide (GRGDS) and galactose ligand (AHG) by RP-HPLC. (A) Representative RP-HPLC chromatograms of arginine (a), hydrolysis product of soluble RGD peptide (b) and soluble galactose ligand (c) as standards, and hydrolysis product of the PET-Hybrid conjugated with RGD peptide and galactose ligand (d); (B) conjugation efficiency curve of RGD peptide onto PET-pAAc; (C) conjugation efficiency curve of galactose ligand onto PET-pAAc.

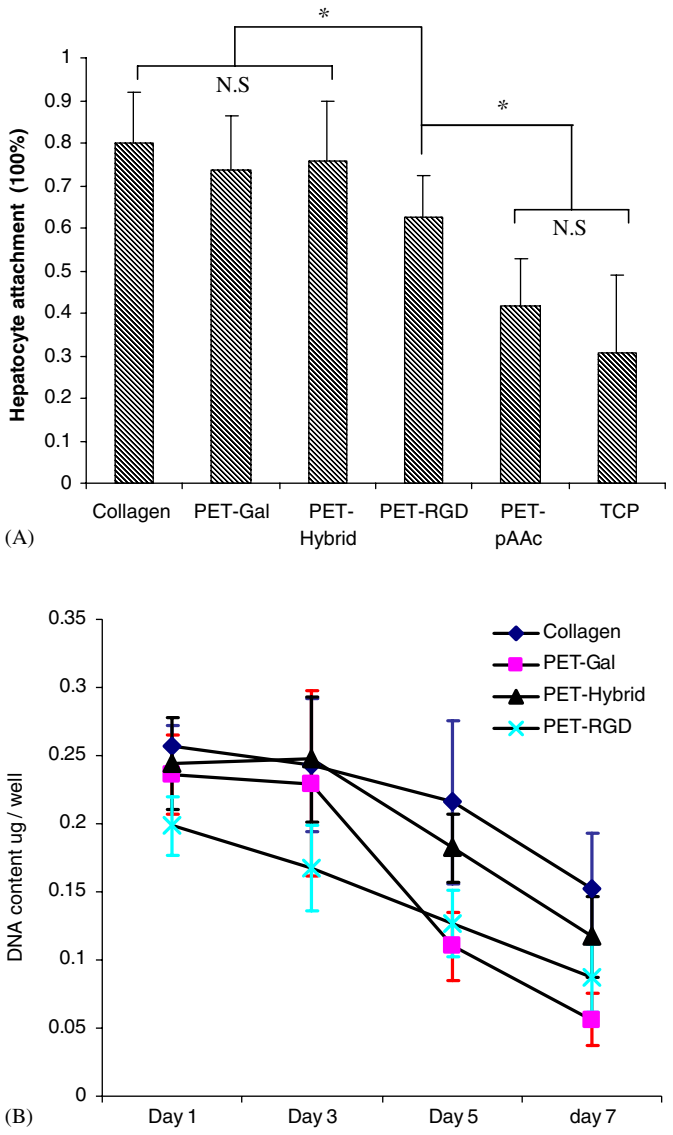

Fig. 4. Hepatocyte attachment to bioactive substrata as represented by the DNA content measurements: (A) hepatocytes attached onto different substrata 2 h after seeding, and (B) at various time points during 7-day culture. Data are means ± SD, $n = 10$. (*): $P < 0.05$, (**): $P < 0.01$, (N.S): not significant.

3.4. Morphological changes of hepatocytes on bioactive substrata over time

Significant influence of substratum characteristics and conjugated bioactive ligands on hepatocyte morphologies was observed. Fig. 5 presented confocal transmission images of the cultured hepatocytes on days 1, 3 and 6 after cell seeding. Within 1 day after seeding, hepatocytes formed small pre-spheroids on PET-Gal and non-spreading aggregates on PET-Hybrid (RGD:galactose = 1:1) while hepatocytes started to spread on PET-RGD and collagen substratum. After 3-day culture, less compact 3D spheroids formed on PET-Gal with some cells detached

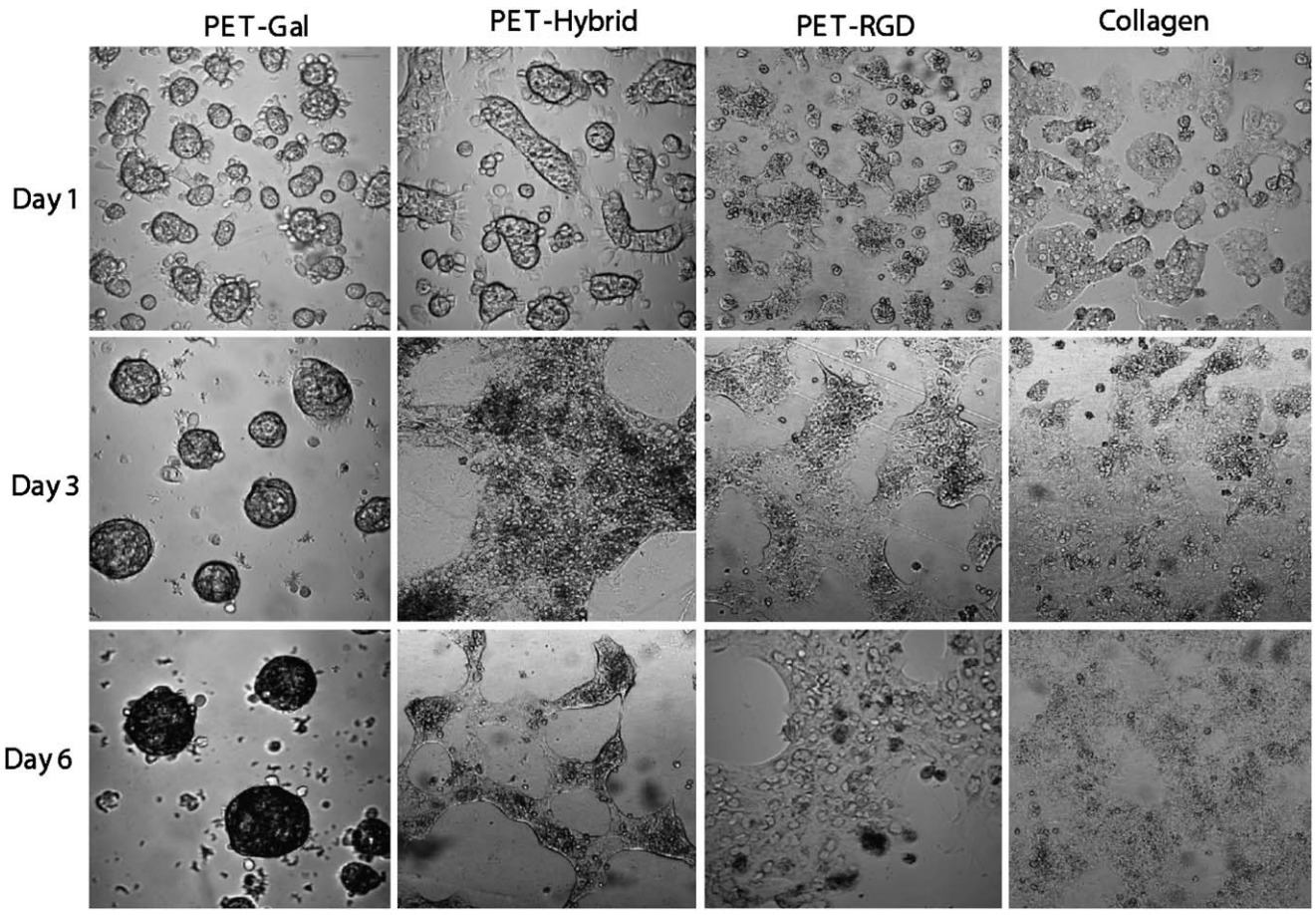

Fig. 5. Confocal transmission images of primary hepatocytes cultured on different substrata at various time points during 7-day culture (scale bar: 50 μm).

from the substratum. Hepatocytes on PET-Hybrid formed limited-spreading thick monolayer with distinct cell–cell boundary. On PET-RGD and collagen substratum, hepatocytes were fully-spread and flattened to form 2D monolayer. On day 6, mature spheroids were observed on PET-Gal and most of which detached from the substratum. Hepatocyte monolayer cultured on PET-Hybrid was stretched to 'island-like' thick monolayer which was distinct from the fully spreading 2D monolayer of hepatocytes cultured on PET-RGD and collagen substratum. This thick hepatocyte monolayer on PET-Hybrid could be maintained for at least 1 week before detachment from the substratum. We named the thick monolayer of hepatocytes cultured on PET-Hybrid as '3D monolayer' which was distinct from the spreading '2D monolayer' seen on PET-RGD and collagen substratum. We did not observe significant morphological differences in hepatocytes cultured on PET-Hybrid with different RGD: galactose ratios (1:5, 1:1, 5:1) (data not shown). This was consistent with the previous findings that RGD density as low as 0.1 pmol/cm^2 was sufficient to induce cell spreading [30]. Therefore, only the PET-Hybrid with 1:1 ratio of RGD:galactose was used in subsequent experiments.

3.5. *F-actin, p-FAK and E-Cadherin distributions in hepatocytes cultured on bioactive substrata*

We hypothesize that the hepatocytes cultured as 3D monolayer on PET-Hybrid experience stronger cell–cell and weaker cell–substratum interactions than the 2D monolayer so as to maintain their 3D cell morphology. The 3D monolayer cultured on PET-Hybrid should also have a stronger cell–substratum interaction than the 3D spheroids on PET-Gal so as to adhere better to the culture substrata as observed above. Actin filament (F-actin) distribution has been used to characterize the relative strength of the cell–cell and cell–substratum interactions experienced by hepatocytes [31,32]. The 2D monolayer of hepatocytes cultured on collagen substratum showed intense actin stress fibers throughout the cells indicating strong cell–substratum interaction (Fig. 6). Hepatocytes cultured as the 3D monolayer on PET-Hybrid had less actin stress fibers than the 2D monolayer on collagen substratum but more stress fibers than the 3D spheroids, indicating an intermediate strength of cell–substratum interaction. The 3D monolayer on PET-Hybrid exhibited cortical F-actin distribution similar to the 3D spheroids cultured on PET-Gal indicating strong cell–cell interaction characteristic of hepatocytes *in vivo* [33].

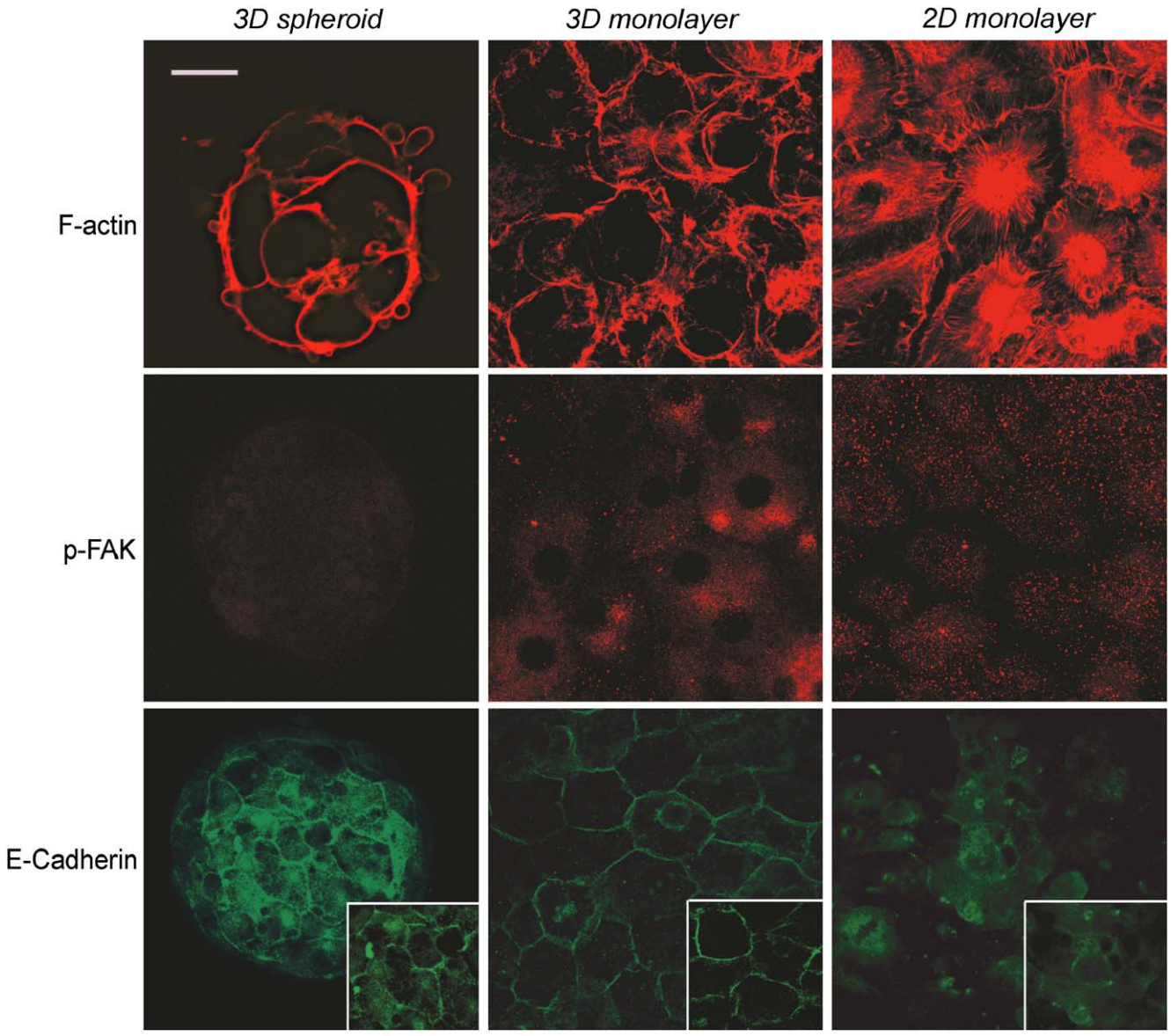

Fig. 6. Confocal images of F-actin, p-FAK and E-Cadherin of hepatocytes after 3-day culture as the 3D spheroid, 3D monolayer and 2D monolayer contains 3D projection of the images (scale bar: 20 μm); E-Cadherin distribution (lower panel) also contains insets of single optical section to confirm whether E-Cadherin localizes at cell boundary.

FAK is a key protein involved in modulating focal adhesion assembly in response to the cell–substratum interaction via integrin [34]. Integrin-mediated cell–substratum adhesion triggers autophosphorylation at the Tyr-397 residue of FAK [35], so that the p-FAK distribution as intracellular clusters is a specific indicator of the cell–substratum interaction. The punctate p-FAK cluster signals were strong in the 2D and 3D monolayers and very weak in the 3D spheroids (Fig. 6) confirming that the 3D monolayer experienced stronger cell–substratum interaction and could adhere better to the substrata than the 3D spheroids.

As a homophilic cell adhesion molecule to regulate recognition and interaction between cells, E-cadherin has been shown to play an important role in the formation of 3D hepatocyte spheroids [36]. We investigated the E-cadherin expression as a specific indicator of cell–cell interaction. E-cadherin was found to localize primarily at the cell–cell boundary at relatively high level in 3D monolayer and spheroids but intracellularly and sparingly throughout the hepatocyte cytoplasm in the 2D monolayer. This confirms the stronger cell–cell interaction in the 3D monolayer and spheroids than the 2D monolayer.

3.6. Hepatocyte functions in response to bioactive substrata

Hepatocytes lost their differentiated functions when stretched as 2D monolayer but exhibited high levels of functions when cultured as 3D spheroids, in 3D microcapsules [37] or in 3D bioreactors [38]. Therefore, it is reasonable to hypothesize that the hepatocytes cultured as the 3D monolayer on PET-Hybrid should exhibit

differentiated functions more similar to the 3D spheroids than the 2D monolayer. Indeed, albumin secretion and urea synthesis by hepatocytes cultured on PET-Gal and PET-hybrid were higher than those on collagen substratum over a 7-day culture with the most significant differences from days 3 to 7. The albumin secretion and urea synthesis by hepatocytes cultured on collagen substratum dropped mostly dramatically even though the functions of hepatocytes cultured on PET-Hybrid and PET-Gal also deteriorated slowly over time (Fig. 7A, 7B). Cytochrome P450 enzymes belong to a class of constitutive and inducible enzymes that metabolize many endogenous substrates, as well as numerous xenobiotics and therapeutic agents including APAP which was the model drug used for hepatotoxicity study [39]. CYP1A is the primary enzyme responsible for the metabolism of EROD and the activity of the enzyme is known to be induced by 3MC. Hepatocytes cultured on all substrata could maintain induced EROD activity over a 7-day culture (Fig. 7C). The induced EROD level was significantly higher in hepatocytes cultured on PET-Gal and PET-Hybrid than on collagen substratum.

3.7. *Response to acetaminophen-induced hepatotoxicity in hepatocytes cultured on bioactive substrata*

APAP, a commonly used analgesic, is known to cause hepatotoxicity when ingested in large quantities in both animals and man, especially when co-administered after chronic ethanol consumption [40]. Hepatotoxicity stems from APAP biotransformation by cytochrome P450 (P450) enzymes to a toxic intermediate which can bind to tissue macromolecules, thereby initiating cellular necrosis [40]. CYP1A, CYP2E and CYP3A are the most active isoforms that can metabolize APAP [41,42]. Induction of CYP activities results in increased APAP toxicity [43]. We investigated the responses to hepatotoxicity caused by APAP itself and co-administration with 3MC to hepatocytes cultured on PET-Gal, PET-Hybrid and collagen substratum. The co-administration of 3MC, an inducer of CYP 1A was conducted as the evaluation of drug–drug interaction which would lead to higher toxicity.

The survival ratio of hepatocytes cultured on different substrata after exposure to APAP or APAP co-administered with 3MC for 24 h (Fig. 8A) or 48 h (Fig. 8B) were measured using an MTS viability assay. In drug-free condition, hepatocytes cultured on all substrata showed similar basal level of viability (data not shown). In the presence of drugs, the 3D monolayer cultured on PET-Hybrid showed similar level of viability as the 3D spheroids cultured on PET-Gal which was more sensitive to hepatotoxicity than the 2D monolayer cultured on collagen substratum. Exposure to low concentration of APAP (2 mM) for 24 h was almost non-toxic to the 2D monolayer (survival ratio of 98%) but slightly toxic to the 3D spheroids (survival ratio of 84%) and the 3D monolayer (survival ratio of 89%); 48 h exposure to 2 mM

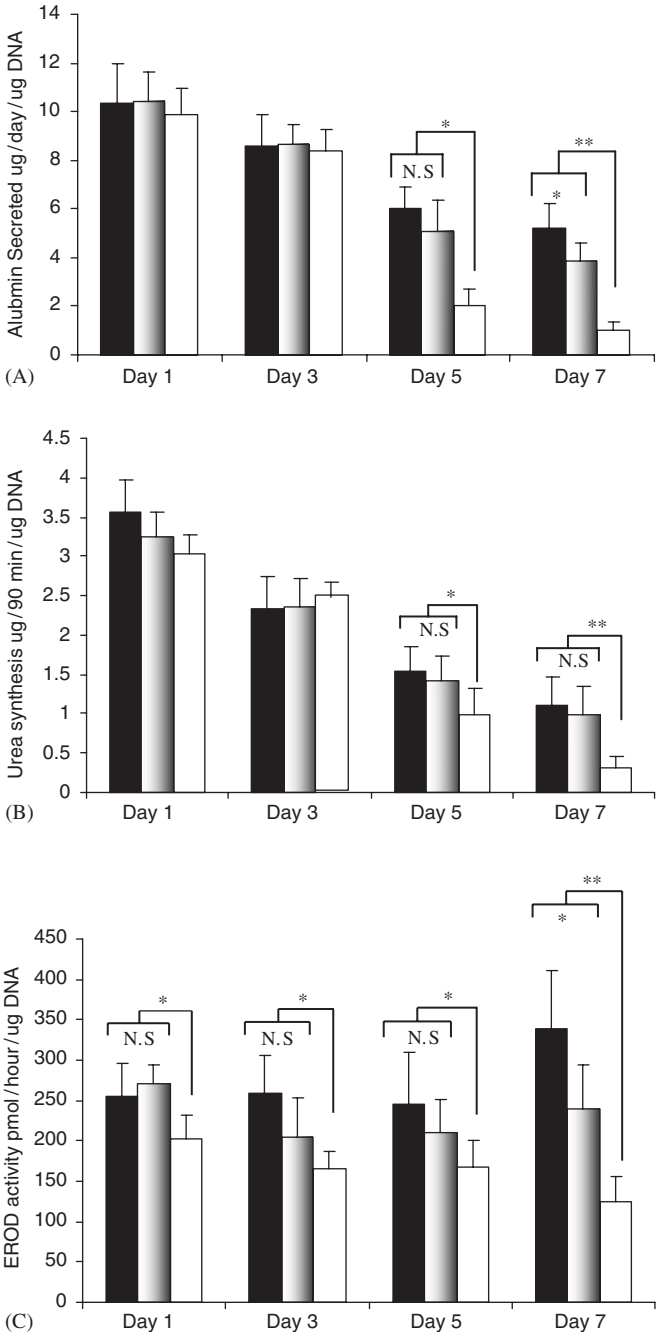

Fig. 7. Liver-specific functions of hepatocytes on different substrata at various time points during 7-day culture: (A) Albumin secretion; (B) urea synthesis and (C) 3MC-induced EROD activity. The functional data were normalized against the DNA content per sample. Data are means ± SD, $n = 6$. (*): $P < 0.05$, (**): $P < 0.01$, (N.S): not significant (■: PET-Gal; ▩: PET-Hybrid; □: collagen).

APAP caused considerable hepatotoxicity to the 2D monolayer (survival ratio of 64%) and more severe toxicity to the 3D spheroids (survival ratio of 57%) and the 3D monolayer (survival ratio of 59%). When exposed to high concentration of APAP (10 mM) for 24 h, the 3D spheroids (survival ratio of 44%) and the 3D monolayer (survival ratio of 38%) showed approximately twice more sensitive

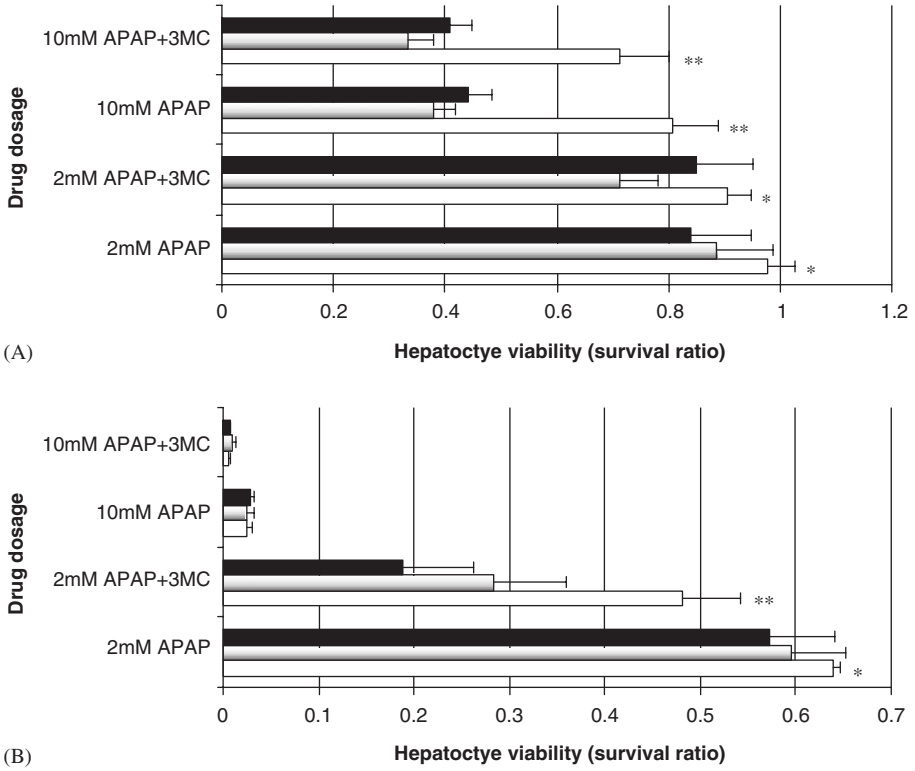

Fig. 8. Response of hepatocytes cultured on different substrata to APAP-induced hepatotoxicity. Survival ratios of hepatocytes exposed to different concentrations of APAP or APAP co-administered with 3MC for 24 h (A) and 48 h (B). Data are means ± SD, $n = 10$. (*): $P < 0.05$, (**): $P < 0.01$, (N.S): not significant (■: PET-Gal; ▩: PET-Hybrid; □: collagen).

to APAP-induced hepatotoxicity than the 2D monolayer (survival ratio of 80%); 48 h exposure to 10 mM APAP killed most of the cells on different substrata. The 'amplified effect of hepatotoxicity' of 3MC when co-administered with APAP became significant only after 48 h exposure. Almost all cells were dead when exposed to 10 mM APAP and 3MC on different substrata. When exposed to 2 mM APAP and 3MC for 48 h, the 3D spheroids (survival ratio of 19%) and the 3D monolayer (survival ratio of 28%) showed approximately 3 times and twice more sensitivity to hepatotoxicity than the 2D monolayer (survival ratio of 48%).

4. Discussion

We have developed a hybrid bioactive substratum for hepatocytes culture which contains both RGD peptide for enhanced attachment, and galactose ligand for maintaining 3D cell morphology and high levels of differentiated functions. Similar combinatorial approach of different bioactive peptides or natural ECM proteins to make biomimetic hybrid-biomaterials have achieved desired regulation of specific cell types [44–46]. The relative strengths of cell–cell versus cell–substratum adhesive interactions ultimately determine the cellular phenotypes [17]. The ligand–receptor interaction between galactose and ASGPR is relatively weak [11]. When primary hepatocytes are cultured on PET-Gal, the adhesive forces from substratum, which is essentially defined by the relatively weak ASGPR-galactose interaction, might not be strong enough to hold the cells down onto the substratum. The hepatocytes thus support each other by establishing strong cell–cell interaction and form 3D spheroids which finally detach from the substratum over time. On the other hand, collagen/RGD on 2D substrata interact strongly with the $\alpha 1\beta 1$ integrin on hepatocytes, which induce downstream signaling to cause cytoskeleton redistribution, focal adhesion complex formation and cell spreading [47]. The hepatocyte adhesion onto collagen/PET-RGD is stronger than the cell–cell interaction. Therefore, the hepatocytes attached to collagen substratum or PET-RGD exhibit fully spreading and flattened morphology as 2D monolayer. ASGPR-galactose interaction has been shown to limit integrin signaling [48]. When primary hepatocytes are cultured on the hybrid substratum with both RGD peptide and galactose ligand, a morphological state named '3D monolayer' has been observed in which hepatocytes contact each other with tight cell–cell interactions and at the same time with effective adhesion to the substratum. Differentiated functions and sensitivity to hepatotoxicity of the 3D monolayer are also similar to the 3D spheroids.

The 3D spheroid-mimetic phenotypes, monolayer culture configuration and effective attachment of the 3D hepatocyte monolayer on the PET-Hybrid substratum

make it a promising alternative for conventional 2D monolayer on collagen substratum for hepatocyte-based applications. Microplate-based metabolism, hepatotoxicity evaluation and flat-plate BLAD all require hepatocytes with high levels of differentiated functions; and 3D monolayer can achieve near the same level as the gold standard, 3D spheroids, and much better than the 2D monolayer control. Hepatocytes cultured on the hybrid-substratum show more sensitivity towards APAP-induced hepatotoxicity than hepatocytes cultured on collagen substratum likely due to the higher CYP450 enzymatic activities. The 'amplified effect of hepatotoxicity' of the co-administered inducer (3MC) might be due to the higher inducibility of CYP 450 enzymes of hepatocytes cultured on PET-Hybrid and PET-Gal. The single layer of hepatocytes in the 3D monolayer ensures good mass transport properties for effective exchange between the cells and the xenobiotics (e.g. drugs) in the culture medium, which is essential for quantitative analysis of the metabolites. The monolayer culture configuration also enables optical microscopy-based assays to be easily implemented to assess xenobiotics metabolism and hepatotoxicity responses directly within living cells. Such advantage can be further exploited to develop high content cell-based screening technologies for various applications [49]. The effective cell attachment to substratum minimizes cell loss during culture and will be important for quantitative analysis of drug–drug interaction which requires a minimum of 3-day culture as mandated by recent FDA ruling [50]. The good chemical, mechanical and optical properties of PET-based substrata also make them ready to be incorporated into the microplate-based high throughput xenobiotics screening system.

5. Conclusions

We have developed a novel hybrid RGD/galactose substratum based on PET film conjugated with a mixture of RGD peptide and galactose ligand to enhance cell adhesion and functions synergistically. Primary hepatocytes adhere effectively onto the transparent hybrid substratum in 96-well plates as monolayer while exhibiting high levels of hepatocyte functions, morphology and cell–cell interactions reminiscent of the cells in 3D spheroids. The hepatocytes cultured on the hybrid substratum also exhibit high sensitivity to APAP similar to the hepatocyte spheroids on galactosylated substratum. The monolayer of hepatocytes exhibiting the 3D cell behaviors on this flat hybrid substratum is compatible with any existing 2D hepatocyte culture platform well-established for high throughput xenobiotics screening, high content cell-based assays and BLAD applications.

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