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In vitro study of the interactions of galactosylated thermo-responsive hydrogels with cells

Ning Yang[a], Li Chen[a,*], Miao-kun Yang[a], Si-xin Bi[a], Xiao-ling He[a], Zheng-yan Zhu[b], Mei-li Yu[c]

[a] Tianjin Key Laboratory of Fiber Modification and Functional Fiber, School of Materials Science and Engineering, Tianjin Polytechnic University, Tianjin 300387, China
[b] Key Laboratory of Regenerative Medicine, Tianjin Third Central Hospital, Tianjin 300170, China
[c] The Institute of Liver and Gall Disease, Tianjin Third Central Hospital, Tianjin 300170, China

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ABSTRACT

The aim of this work is to explore the ability of poly(N-isopropylacrylamide) (NIPAAm) with galactosylated acrylate (GAC) hydrogels as cell scaffolds for HL-7702 cells to proliferate, keep function of albumin and urea synthesis and detach. The results indicate poly(NIPAAm-co-GAC) hydrogels with weight ratio of 0.2 could support cells to adhere, proliferate and maintain their liver functions for 7 days. An intact cells sheet could spontaneously detach from poly(NIPAAm-co-GAC) ($r = 0.2$) hydrogels without treating with any enzymes. And detached cells show more integrate morphology (H&E staining) and higher proliferation index (cell cycle analysis) than cells detached by trypsin treatment. The results also reveal excessive GAC introduced to PNIPAAm hydrogels could induce apoptotic cell death of adhered cells.

1. Introduction

Hydrogels are cross-linked hydrophilic polymers that can absorb water or biological fluids and swelling. Due to the high level of water in composition and elastic structure, hydrogels are considered as excellent biocompatible materials (Satarkar & Zach Hilt, 2008). In recent years, intelligent hydrogels which can change their swelling behavior and other properties in response to small environmental stimuli such as temperature, pH, ionic strength etc. have attracted considerable attention (Byrne & Salian, 2008; Frisman, Shachaf, Seliktar, & Bianco-Peled, 2011; He, Zuo, Xie, Huang, & Xue, 2011; Kanazawa, 2007; Prabaharan & Mano, 2006; Shofner, Phillips, & Peppas, 2010; Xiao et al., 2009). Among them, polymer hydrogels with thermo sensitivity such as PNIPAAm hydrogels in particular exhibit a clear coil-to-globule transition at its lower critical solution temperature (LCST = 32 °C) become the research hotspot. Polymer chains become fully hydrated and show extended chain conformation below the LCST and dehydrated and shrunken above the LCST. This unique property of poly(N-isopropylacrylamide) (PNIPAAm) to switch between hydrophilic and hydrophobic character in ambient temperature range could be used in controlled drug delivery, bioengineering or tissue engineering (Song, Wang, & Wang, 2011; Tekin et al., 2011).

Using chemical reaction or radical graft polymerization (RGP) method, PNIPAAm were grafted onto tissue culture-polystyrene dishes (TCPS) by Okano's group (Elloumi-Hannachi, Yamato, & Okano, 2010; Fukumori et al., 2010; Okano, Yamada, Sakai, & Sakurai, 1993; Okano, Yamada, Okuhara, Sakai, & Sakurai, 1995; Yamato et al., 2001). Cultured cells in vitro could adhere to a slightly hydrophobic surface that provided by PNIPAAm coated surfaces at culture conditions of 37 °C, cells could proliferate to a single continuous cell sheet further. The modification surfaces facilitate the detachment of cells by lowering temperature below the LCST without using proteolytic enzymes (Nishi et al., 2007). They developed a novel technique of cell-sheet engineering for tissue reconstructions (Elloumi-Hannachi et al., 2010). However, the PNIPAAm grafted TCPS show poor reswelling ability which is not suitable for spontaneous cell sheet detachment. It is known that hydrogels has porous conformation, and just this porous conformation could accelerate the hydration of the material. When temperature decreased from 37 °C to 20 °C, reswelling of thermo-sensitive hydrogels occurred due to the hydration of PNIPAAm. The reswelling of hydrogels supplied water molecules not only from the periphery of each cell but also through pores underneath the adherent cells. Furthermore, incorporation of a highly water permeable substrate to interface between the cell sheet and the thermo responsive polymer surfaces could accelerate the hydration of the hydrophobized PNIPAAm segments interacting with the cell sheet. So the

* Corresponding author. Tel.: +86 22 24528013; fax: +86 22 24528013.
E-mail addresses: chenlis@tjpu.edu.cn, tjpuchenlis@163.com, summer.ningzi@yahoo.com.cn (L. Chen).

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polysaccharide modified PNIPAAm hydrogels is an important vehicle for accelerating spontaneous cell sheet detachment.

Galactose is known as a specific adhesive ligand to asialoglycoprotein receptor (ASGR) of hepatocytes (Chung et al., 2002; Park et al., 2003; Yang, Goto, Ise, Cho, & Akaike, 2002). Our laboratory have previously synthesized galactosylated acrylate (GAC) hydrogels with NIPAAm, and its swelling behavior, temperature-sensitivity and cyto-biocompatibility of poly(NIPAAm-co-GAC) hydrogels were investigated as well (He et al., 2010; Wang et al., 2010). In present work, the interactions between poly(NIPAAm-co-GAC) hydrogels and a normal human liver cells line (HL-7702 cells) were further studied. The bio-function of poly(NIPAAm-co-GAC) hydrogels was first discussed in this paper. And particularly, the advantages of spontaneous detachment to trypsin digestion were proved by the tests of cell morphology observation and cell cycle analysis. An intact hepatocytes sheet was harvested from thermo-responsive hydrogels. We expected it could be used in liver tissue engineering or bio-artificial liver devices in future.

2. Experimental

2.1. Materials

NIPAAm obtained from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan) and purified by recrystallization from toluene and *n*-hexane. Acrylic acid purchased from Tianjin Institute of Chemical Reagents (Tianjin, China) and purified by distillation under reduced pressure. N-(3-dimethylaminopropyl)-N-ethylcar-bodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide sodium salt (sulfo-NHS) were the products of Shanghai Med pep Co. Ltd. (Shanghai, China). Ammonium persulfate (APS), N,N,N′,N′-tetra-methylenediamine (TEMED), and N,N′-methylenebis(acrylamide) (MBAA) were analytical grade made in China. HL-7702 cells were kindly provided by Tianjin Third Central Hospital (Tianjin, China). Phosphate-buffered saline (PBS, pH 7.2–7.4) used for hydrogels sterilization. Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (Minhai biotechnologies), penicillin and streptomycin, trypsin and ethylenediaminetetraacetic acid (EDTA) were obtained from Shanghai Sangon Biological Engineering Technology & Services Co. Ltd. (Shanghai, China). Water was deionized by Millipore Direct-Q apparatus.

2.2. Synthesis of poly(NIPAAm-co-GAC) hydrogels

GAC was synthesized according to the method previously report (He et al., 2010; Wang et al., 2010). Poly(NIPAAm-co-GAC) hydrogels prepared by the free radical copolymerization of NIPAAm and GAC in aqueous solution. NIPAAm and GAC were dissolved in deionized water according to a weight ratio, in the feed (GAC/(GAC + NIPAAm)) of 0, 0.2 and 0.4, respectively, and then initiator APS (0.2 wt%) and accelerant TEMED (0.5 wt%) were added. After bubbling with nitrogen gas to get rid of oxygen while stirred by vortex for 10 min, the mixture was injected into the glass moldand and let it react at room temperature for 48 h. The formed hydrogels were cut into discs with a diameter of approximately 9 mm and immersed in an excess of distilled water for 1 week with daily water exchange to remove the unreacted monomer (Fig. 1).

2.3. Cells proliferation on thermo-responsive hydrogels

HL-7702 cells were seeded onto different hydrogels at a density of 2×10^4 cells/well and cultured for 7 days. Cells morphology observed under microscopy (OLYMPUS IX71, Japan) and MTT assay used to quantify metabolic activity in each well. At desired time, the culture medium was drained and re-supplied with 1 mL of fresh culture medium that containing MTT (0.5 mg/mL). After incubating at 37 °C for 4 h, excess medium was removed and added 500 μl DMSO to dissolve the formazan crystal. Then 150 μl DMSO solutions were injected into 96-well plates and OD value of each well was determined by Auto microplate reader (Multiskan EX, Fortune labsystems, CA) while the wavelength was selected at 492 nm.

2.4. Measurement of HL-7702 cells functions

The amounts of albumin and urea in the culture medium were determined by chemiluminescence. Firstly, the collected medium samples were concentrated. Secondly, the albumin concentration was analyzed using bromocresol green and the urea concentration was determined via its specific reaction with urease. The absorbance measured by an auto chemistry analyzer (BECKMAN, CA).

2.5. Detachment of HL-7702 cells sheet

HL-7702 cells were seeded onto poly(NIPAAm-co-GAC) ($r = 0.2$) hydrogels at a final cell density of 2×10^5 cells/well and cultured for one week to obtain confluent cells sheet under a humidified atmosphere of 5.0% CO_2 at 37 °C. The photograph of detached cells sheet was taken by a digital camera (FUJIFILM, Tokyo) and by phase-contrast microscopy (OLYMPUS IX71, Japan).

2.6. Cell cycle distribution of HL-7702 cells

The cell cycle distribution of cultured HL-7702 cells subsequent to materials' cellular compatibility was investigated using flow-activated cell sorter (FACS, BECKMAN, CA) and EPICS ALTRA Software (Applied Cytometry Systems) analysis. Briefly, following inoculation cells on TCPS, PNIPAAm ($r = 0$), poly(NIPAAm-co-GAC) ($r = 0.2$) and poly(NIPAAm-co-GAC) ($r = 0.4$) hydrogels and cultured for specific time. 10^6 cells were trypsinized from TCPS and washed twice with cold PBS and 10^6 cells were collected from thermo responsive hydrogels by cooling detachment. The cells were then stored in 1 mL of 70% alcohol/PBS at −20 °C for subsequent experimentation. For FACS analysis, the cells were centrifuged at 6000 rpm for 5 min, and subsequently washed with PBS, following which they were incubated with 0.5 mL 0.5% Triton X-100/PBS and 10 μl 10 g/L RNaseA for 30 min. The cells were then stained with 20 μl 2.5 g/L propidium iodide/PBS in the dark and analyzed using FACS. The cell cycle distribution of HL-7702 cells following different hydrogels cultured was then assessed. The sub-G_1/G_0 cell fraction was considered as representative of apoptotic cells.

$$\mathrm{PI\,(proliferation\ index)}\,(\%) = \frac{S + G_2/M}{G_0/G_1 + S + G_2/M} \times 100 \qquad (1)$$

Note: G_0/G_1 representative of DNA presynthetic phase, S representative of DNA synthesis phase and G_2/M representative of DNA postsynthetic phase.

2.7. Statistical analysis

All the obtained data were expressed as the mean value with standard deviation. Analysis of variance (ANOVA) of data was carried out with SPSS V13.0 software. The homoscedasticity between two independent experimental groups was confirmed by *F*-test method firstly. Then, homoscedastically guaranteed combinations of independent experimental groups were analyzed by unpaired Student's *t*-test method, respectively. Statistical significance was determined and accepted at $P < 0.05$,obvious significance was determined and accepted at $P < 0.01$.

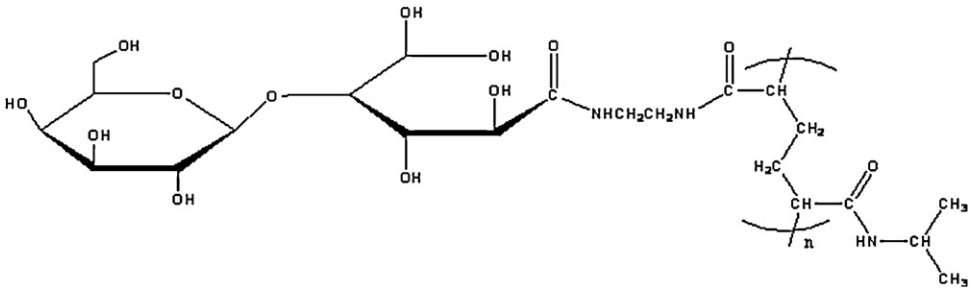

Fig. 1. Structure of poly(NIPAAm-co-GAC).

3. Results and discussion

3.1. Thermo-responsive properties of poly(NIPAAm-co-GAC) hydrogels

The swelling behavior and temperature-sensitivity of poly(NIPAAm-co-GAC) hydrogels in distilled water and cell culture medium were studied by both gravimetric method and DSC. It is found that poly(NIPAAm-co-GAC) hydrogels still exhibit good temperature sensitivity and better swelling properties compared with pure PNIPAAm hydrogel (Wang et al., 2010). Further, the thermo-responsive property of poly(NIPAAm-co-GAC) hydrogels was investigated by measuring the static water contact angle at 25 °C and 37 °C. All surfaces of thermo-responsive hydrogels showed hydrophilic/hydrophobic property alterations with temperature changes between 37 °C and 25 °C (Fig. 2). The temperature sensitivity was improved with increasing NIPAAm content. For example, the difference of contact angle between 25 °C and 37 °C is 18° for poly(NIPAAm-co-GAC) (r = 0.4) and 24° for poly(NIPAAm-co-GAC) (r = 0.2) hydrogels, respectively. The most hydrophobic surface was PNIPAAm hydrogels at 37 °C, while the most hydrophilic surface was poly(NIPAAm-co-GAC) (r = 0.4) hydrogels at 25 °C. In addition, the hydrogels surface hydrophilicity increased with increasing GAC content.

3.2. Cells proliferation on thermo-responsive hydrogels

Cells could proliferate on all surfaces, but have very different morphology (Fig. 3). The most obvious difference could be found on the 7th day (Fig. 3C, F, I and L). More dead cells appeared in Fig. 3C, that may due to the decrease of migration ability of cells. It is noteworthy that cells cultured on PNIPAAm and poly(NIPAAm-co-GAC) (r = 0.2) hydrogels were various. The connections between cell–cell that proliferated on PNIPAAm hydrogels were loose (Fig. 3E and F), while the ones on poly(NIPAAm-co-GAC) (r = 0.2) hydrogels were tight and all confluent cells connected to a single contiguous cells sheet (Fig. 3H and I). The growth speed of HL-7702 cells that proliferate on poly(NIPAAm-co-GAC) (r = 0.4) hydrogels was very slow and the morphology of cells was not as good as cells grew on PNIPAAm or poly(NIPAAm-co-GAC) (r = 0.2) hydrogels. That may influenced by the irregular surface morphology of poly(NIPAAm-co-GAC) (r = 0.4) hydrogels.

Proliferation curves indicated that cells on poly(NIPAAm-co-GAC) (r = 0.2) hydrogels grew more rapidly than that on poly(NIPAAm-co-GAC) (r = 0.4) and PNIPAAm hydrogels during 7 days of incubation (Fig. 3M). Some differences were, however, found that HL-7702 cells did not proliferate on poly(NIPAAm-co-GAC) (r = 0.4) hydrogels during the first 5 days and cells viability had a little decreased on the 3th day. We could speculate that HL-7702 cells were very sensitive for culturing on poly(NIPAAm-co-GAC) (r = 0.4) hydrogels. The results of cell proliferation showed that poly(NIPAAm-co-GAC) (r = 0.2) hydrogels are more suitable for HL-7702 cells to proliferate.

3.3. Apoptosis of HL-7702 cells

The apoptosis was described as the process of programmed cell death in multicellular organisms. If biomaterial is not suitable for cells to adhere and proliferate, apoptosis may happen in these cells. Apoptosis of HL-7702 cells tested using FACS (BECKMAN, CA) to verify the biocompatibility of poly(NIPAAm-co-GAC) (r = 0.4) hydrogels.

The cell cycle modifications after 24 h, 48 h or 72 h of post-incubation on poly(NIPAAm-co-GAC) (r = 0.4) hydrogels were

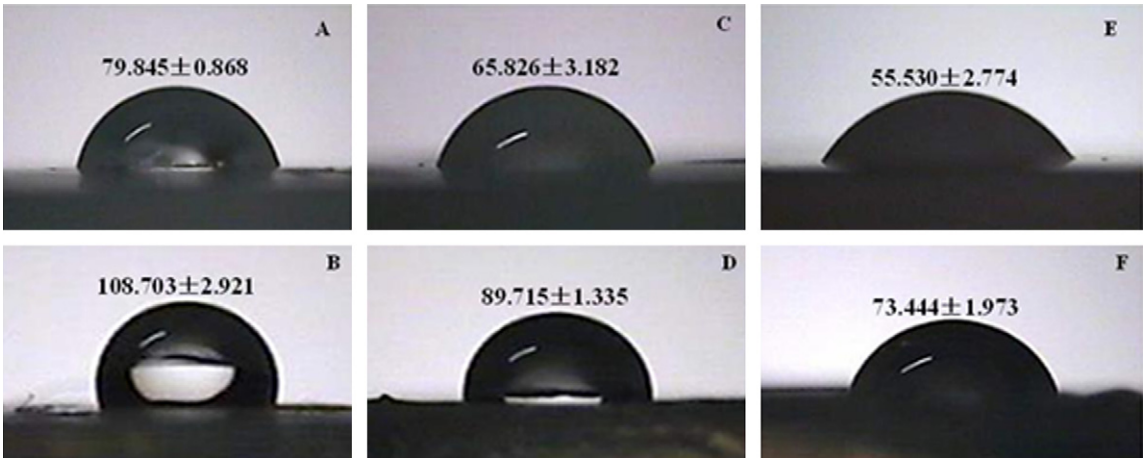

Fig. 2. The contact angle of thermo-responsive hydrogels on surfaces. (A) PNIPAAm (r = 0), 25 °C; (B) PNIPAAm (r = 0), 37 °C; (C) poly(NIPAAm-co-GAC) (r = 0.2), 25 °C; (D) poly(NIPAAm-co-GAC) (r = 0.2), 37 °C; (E) poly(NIPAAm-co-GAC) (r = 0.4), 25 °C; (F) poly(NIPAAm-co-GAC) (r = 0.4), 37 °C.

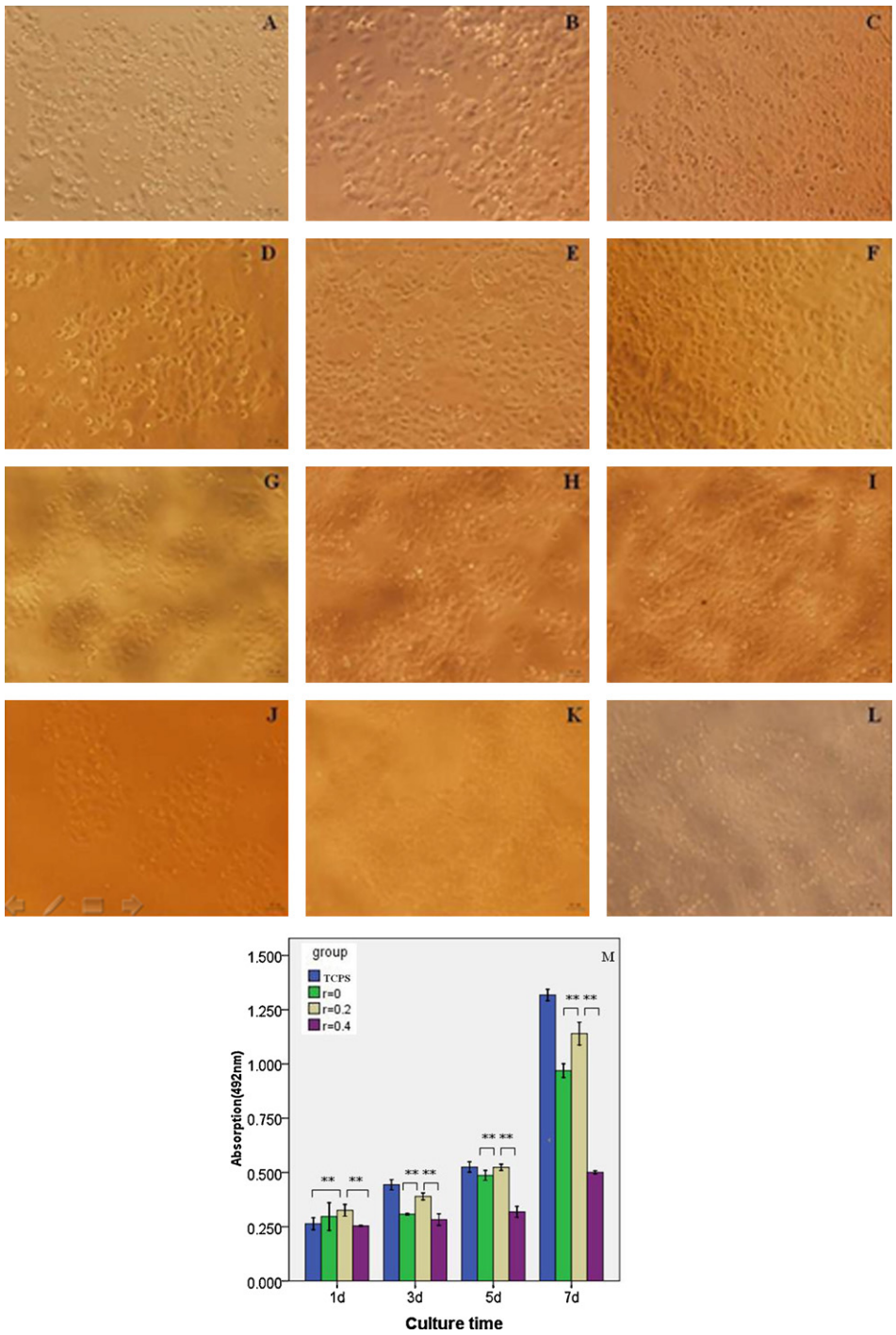

Fig. 3. HL-7702 cells proliferation. Cells cultured on different surfaces on 1d, 5d and 7d for TCPS (A)–(C); PNIPAAm (D)–(F); poly(NIPAAm-co-GAC)(r = 0.2) (G)–(I); poly(NIPAAm-co-GAC) (r = 0.4) (J)–(L). Scale bars: 50 μm; (M) proliferation curves. The same cell density (2×10^4 cells/well) seeded onto different hydrogels and cultured for 1d, 3d, 5d and 7d. At prescribed time, MTT solution (5 mg/mL) added and cultured for 4 h. DMSO dissolved the crystallization purple and OD value of each well was determined by auto microplate reader while the wavelength selected at 492 nm.

Table 1
Apoptosis rate and cell cycle of cells.

Group	Apoptosis rate (sub-G_0/G_1)	G_0/G_1	S	G_2/M
Control	4.787 ± 0.680	66.579 ± 2.377	22.329 ± 0.396	10.200 ± 1.297
r = 0.4–24 h	6.926 ± 0.085**	63.119 ± 0.876**	22.990 ± 1.777	13.702 ± 1.395*
r = 0.4–48 h	7.780 ± 0.108**	77.227 ± 1.115**	12.398 ± 3.868**	10.375 ± 3.128
r = 0.4–72 h	15.107 ± 0.152**	71.970 ± 0.917**	16.928 ± 3.174**	11.102 ± 2.257

* P < 0.05 vs. Control.
** P < 0.01 vs. Control.

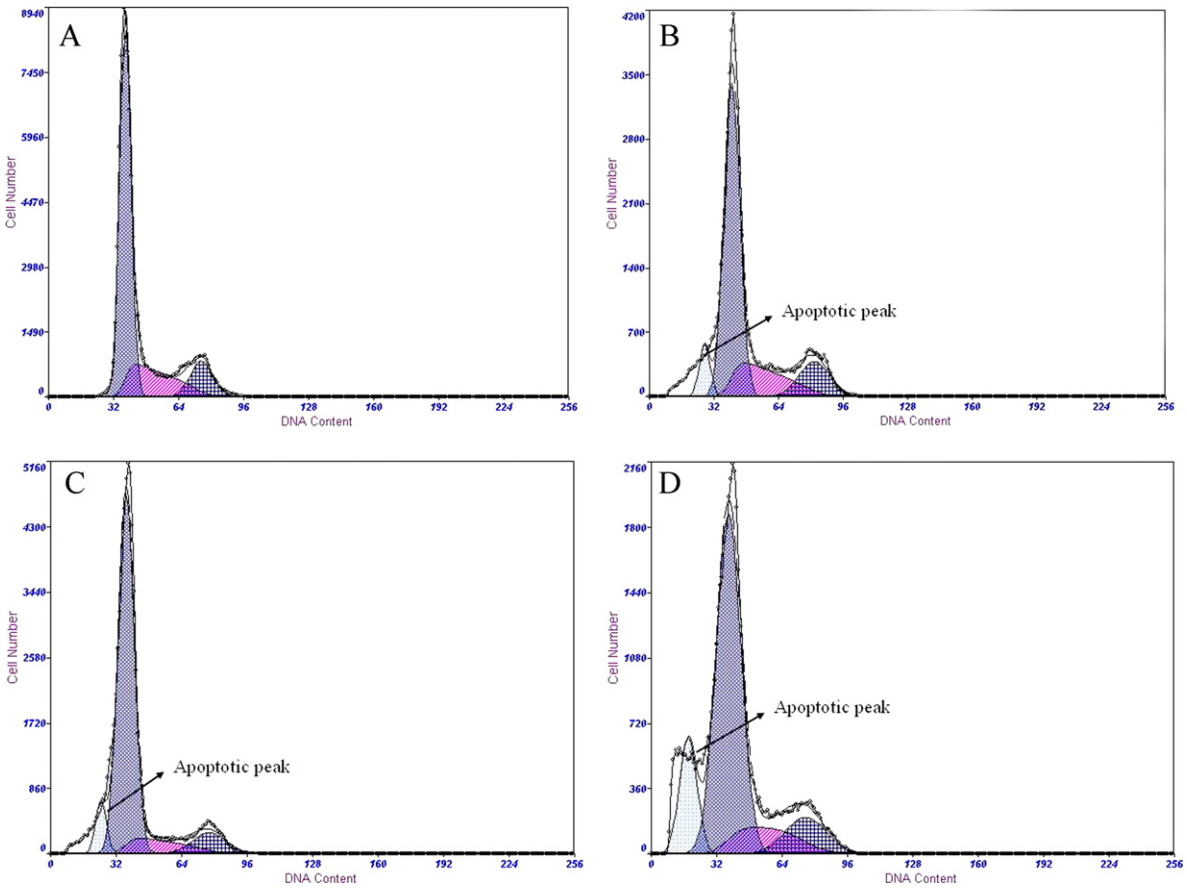

Fig. 4. Analysis of cell cycle and apoptosis. Inoculation cells on poly(NIPAAm-co-GAC) ($r = 0.4$) hydrogels and cultured for 24 h, 48 h and 72 h. 10^6 cells were collected from thermo responsive hydrogels by cooling detachment. The cells were then analyzed using FACS for apoptosis detected. (A) Cells before inoculation used as control; (B) cells cultured on poly(NIPAAm-co-GAC) ($r = 0.4$) hydrogels for 24 h; (C) cells cultured on poly(NIPAAm-co-GAC) ($r = 0.4$) hydrogels for 48 h; (D) cells cultured on poly(NIPAAm-co-GAC) ($r = 0.4$) hydrogels for 72 h.

assessed (Table 1). HL-7702 cells that before inoculation used as control. The flow cytometry graphs of cell cycle were shown in Fig. 4. The most notable change of cell cycle appeared in HL-7702 cells that incubated on poly(NIPAAm-co-GAC) ($r = 0.4$) hydrogels for 48 h and 72 h ($P < 0.01$) (Fig. 4C and D). Predominant decrease of cells in S-phase ($P < 0.01$ vs. control) was observed, whereas cells significant accumulated in the G_0/G_1-phase (Table 1) that indicated HL-7702 cells were injury. The apoptotic rate of HL-7702 cells abruptly increased with time delayed, meanwhile have obvious difference ($P < 0.01$) with control. We could inferred that poly(NIPAAm-co-GAC) ($r = 0.4$) hydrogels had low cell compatibility. When HL-7702 cells planted and kept on proliferation on poly(NIPAAm-co-GAC) ($r = 0.4$) hrdrogels, a fraction cells induced to happen apoptotic cell death. That may attribute to GAC was anionic polysaccharide, and the hydrogels surface hydrophilicity increased with increasing GAC content (Fig. 2). However, according to other research (Brodbeck et al., 2001, 2002; Gretzer, Gisselfält, Liljensten, Rydén, & Thomsen, 2003) surfaces displaying strongly

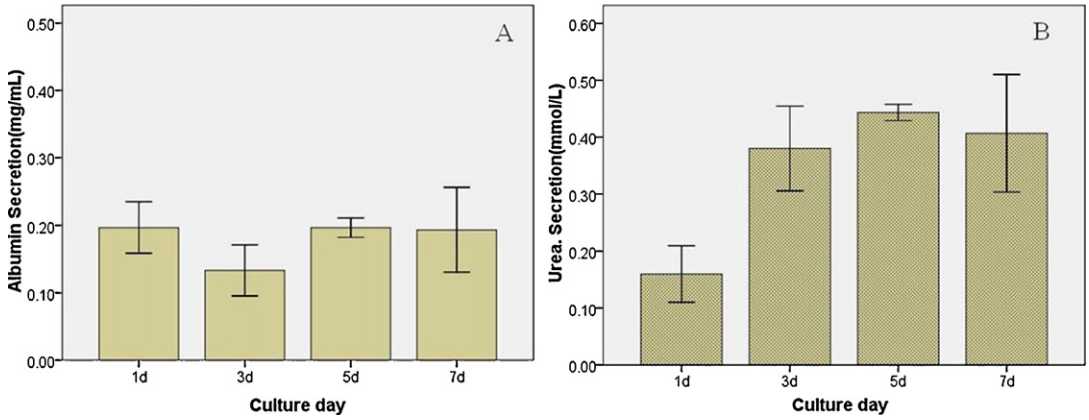

Fig. 5. Cells ability of albumin and urea synthesis. HL-7702 cells cultured on poly(NIPAAm-co-GAC) ($r = 0.2$) hydrogels for 7 days. Culture supernatant collected at indicated time and tested for albumin secretion and urea synthesis through auto chemistry analyzer (BECKMAN, CA). (A) Cells ability of albumin secretion; (B) cells ability of urea synthesis.

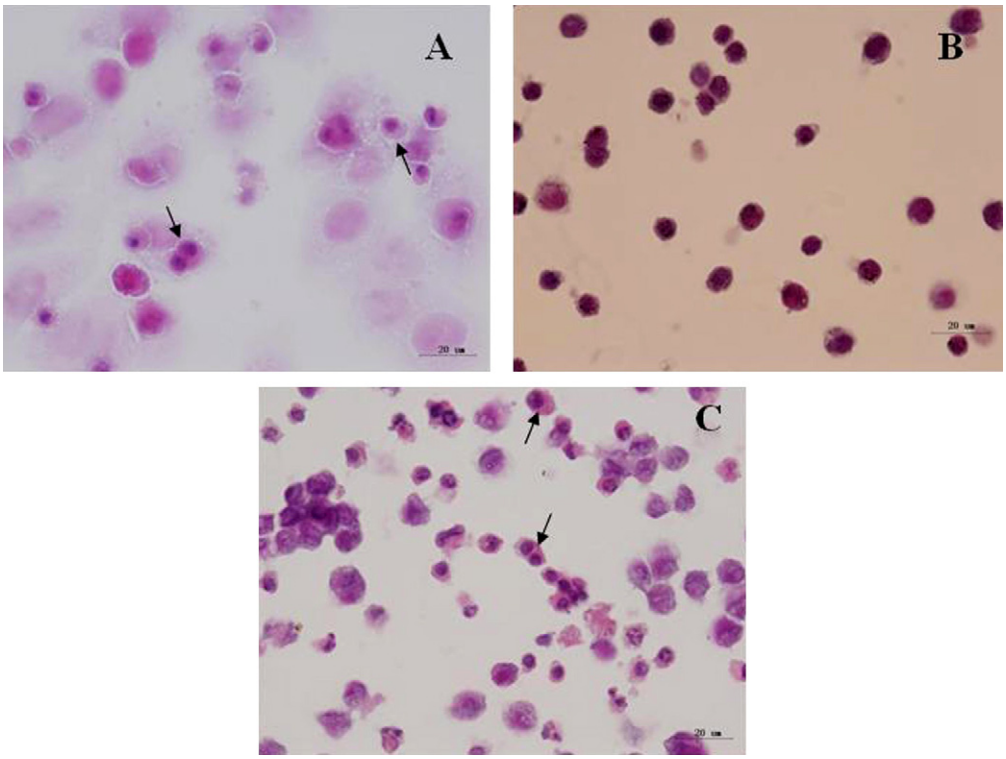

Fig. 6. H&E staining of detached cells. Cells were collected from different methods of detachment. They were then stained with H&E by conventional methods and observed under microscope. (A) Cells of control culture; (B) cells detached from TCPS by trypsin digestion; (C) cells detached from poly(NIPAAm-co-GAC) ($r = 0.2$) hydrogels by reducing temperature. Cells that marked with arrows have similar morphology. Scale bars: 20 μm.

hydrophilic and anionic chemistries could induce apoptosis of adherent cells. No apoptosis of HL-7702 cells that proliferated on poly(NIPAAm-co-GAC) ($r = 0.2$) or PNIPAAm hydrogels were observed. So poly(NIPAAm-co-GAC) ($r = 0.4$) hydrogels were not suitable for HL-7702 cells to adhere and proliferate (Fig. 3).

3.4. The biosynthetic activity of HL-7702 cells

Albumin and urea synthesis was the important functional capacity of hepatocytes. The above experiment results (Fig. 3) verified HL-7702 cells could proliferate on poly(NIPAAm-co-GAC) ($r = 0.2$) hydrogels very well. So we will further discuss whether cells could keep the function of protein synthesis that by culturing on thermo-responsive hydrogels for 7 days. It could be found that the albumin secretion continuously after HL-7702 cells grew on poly(NIPAAm-co-GAC) ($r = 0.2$) hydrogels for 7 days (Fig. 5A). The function of urea synthesis in HL-7702 cells was increased with time delayed from (0.16 ± 0.02) μmol/mL to (0.41 ± 0.04) μmol/mL (Fig. 5B). The results further proved that poly(NIPAAm-co-GAC) ($r = 0.2$) hydrogels with good biocompatibility and bio-function, could be applied in bio-artificial liver.

3.5. Hematoxylin and eosin staining of detached cells

Morphological changes of cells detached from thermo-responsive hydrogels or tryptic digesting were evaluated by hematoxylin and eosin (H&E) staining. First, cells were collected from different methods of detachment. They were then stained with H&E by conventional methods and dehydrated through a graded ethanol series to xylene, and dried in air.

Some significant morphological changes could be found after proteolytic enzymes digesting (Fig. 6B) compared with normal proliferating HL-7702 cells (Fig. 6A). Nuclear and cytoplasm could not be distinguished due to concentration of cytoplasm. Severe damage subjected to HL-7702 cells after trypsin treated. Extracellular matrix proteins and trans-membrane proteins always hydrolyzed by trypsin digested. Communication that between cell–cell and cell–extracellular matrix may be destroyed with trypsin digested. No obvious morphological transforms were found in cells that recovery by lowering temperature detached from

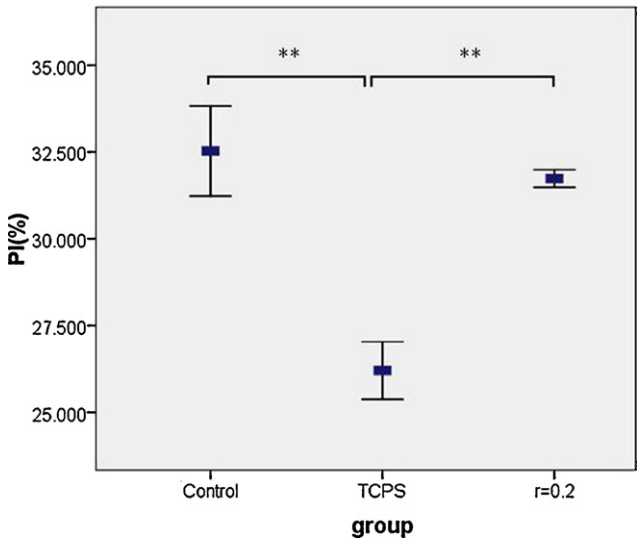

Fig. 7. Proliferation index of detached cells by lowering temperature or tyrpsin digestion. Inoculation cells onto TCPS or poly(NIPAAm-co-GAC)($r = 0.2$) hydrogels and cultured for 72 h. Cells before inoculation used as control. 10^6 cells were trypsinized from TCPS and 10^6 cells were collected from thermo responsive hydrogels by cooling detachment. The cells were then stained with propidium iodide/PBS in the dark and analyzed using FACS. The cell cycle distribution following different methods of detachment was then assessed. $^{**}P < 0.01$. PI (proliferation index) $(\%) = (S + G_2/M)/(G_0/G_1 + S + G_2/M) \times 100$.

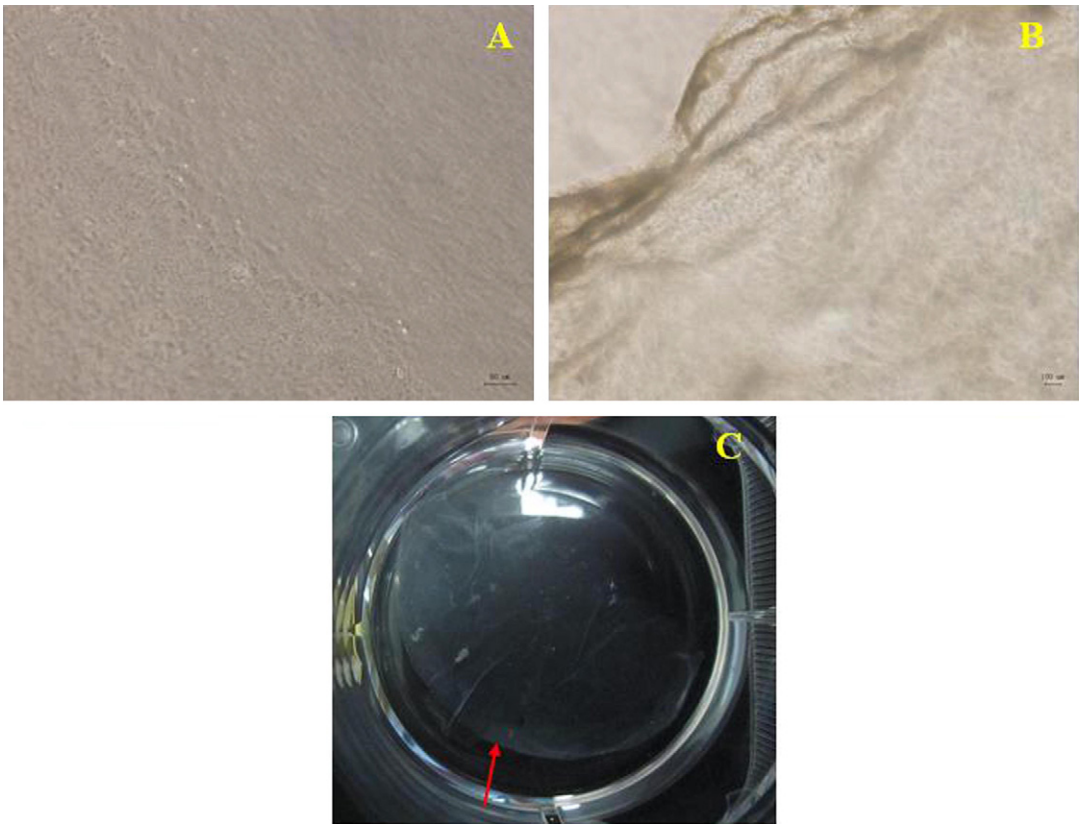

Fig. 8. Cells sheet recovery by lowering temperature. Cells reached confluent when cultured on poly(NIPAAm-co-GAC) ($r = 0.2$) hydrogels for 7 days. By lowering temperature to 20 °C for 30 min, all confluent cells detached as a single contiguous sheet from thermo-sensitive hydrogels. (A) HL-7702 cells reached confluent, scale bars: 50 μm. (B) The fringe of a single contiguous cells sheet, scale bars: 100 μm. (C) A single contiguous cells sheet.

thermo-responsive hydrogels ($r = 0.2$) (Fig. 6C) in comparison to normal cells (Fig. 6A) and cells had very similar morphology in arrows. HL-7702 cells cultured and cooling detached from thermo responsive hydrogels had advantage to cells that digested by trypsin, which was judged from morphological observation. It could be inferred that cooling detached cells had higher cell viability.

3.6. Proliferation index (PI) of detached cells

The proliferative abilities of detached cells were further detected by flow cytometric assay (Fig. 7). Analysis of cell cycle could reflect the proliferative state of the cell population more accurately. The number of cells in S-phase and G_2/M-phase represent the proliferating cells. The PI of HL-7702 cells that before inoculation to different culture surfaces used as control. PI of cells detached from TCPS by tyrpsin digested significant lower ($^{**}P < 0.01$) than control cells (Fig. 7). While the PI of cells detached from poly(NIPAAm-co-GAC) ($r = 0.2$) hydrogels had no difference ($P > 0.05$) with control cells, meanwhile significant higher than cells by tyrpsin digested ($P < 0.01$). These results indicated HL-7702 cells that detached from poly(NIPAAm-co-GAC) ($r = 0.2$) hydrogels had higher proliferation activity than cells detached by tyrpsin digested.

3.7. Cells detachment from thermo-responsive hydrogels

Cells reached confluent when cultured on poly(NIPAAm-co-GAC) ($r = 0.2$) hydrogels for 7 days (Figs. 3I and 8A). By lowering temperature, all confluent cells detached as a single contiguous sheet from thermo-sensitive hydrogels (Fig. 8B and C). Then put the cells sheet to another new well, a large integrity cells sheet recovery by lowering temperature to 20 °C for 30 min (Fig. 8C). Conventionally, cells are harvested using chelating agents such as EDTA to disrupt cell–cell junctions as well as proteolytic enzymes such as trypsin or dispase to free cells from ECM attached to the culture surfaces. Under such processes, there is destruction of ECM, cellular junctions and cell membrane proteins such as ion channels and growth factor receptors. The bio-function of cells was badly destructed (Castner & Ratner, 2002; Waymouth, 1974). By use of thermo-responsive hydrogels to create an intact hepatocytes sheet and tremendous amounts of cells that have good bio-function can be applied in bio-artificial liver or liver tissue architectures reconstruction may soon become possible.

4. Conclusions

In this work, we further studied the interactions between HL-7702 cells and thermo-responsive hydrogels. GAC and a series of poly(NIPAAm-co-GAC) hydrogels were successfully synthesized. It was found that poly(NIPAAm-co-GAC) ($r = 0.2$) hydrogels with best biocompatibility and bio-function. It could support HL-7702 cells to proliferate and keep their function of albumin and urea synthesized at the same time (Figs. 3 and 5). And we also proved that HL-7702 cells apoptosis induced by excessive GAC in hydrogels (Fig. 4). The proliferation state of cells detached from poly(NIPAAm-co-GAC) ($r = 0.2$) hydrogels was revealed by cell cycle analysis (Fig. 7) and this result further proved that cells did not damaged by lowering temperature detachment (Fig. 6C). A large integrity cells sheet recovery from poly(NIPAAm-co-GAC) ($r = 0.2$) hydrogels by lowering temperature treatment (Fig. 8). Conclusively, poly(NIPAAm-co-GAC) ($r = 0.2$) thermo-sensitive hydrogels was optimum to culture HL-7702 cells. It has the potential value to be used in liver tissue engineering or bio-artificial liver devices.

Acknowledgments

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