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ORIGINAL ARTICLE

Carbohydrates–chitosan composite carrier for Vero cell culture

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Abstract In this study, carbohydrate–chitosan composite including glucose–chitosan, sucrose–chitosan and starch–chitosan with varied carbohydrate concentrations were prepared as carriers for Vero cell culture. Our results show that among these composites, 30 % starch–chitosan composite (STC) were the best carriers for the growth of Vero cells. The initial number of attached cells on the surface of composite carriers did not have any significant effect on subsequent cell production. A higher glucose level in the growth medium during the exponential phase of cell growth, however, played an important factor for cell production. Vero cells on the STC carriers were able to convert starch inside the composite carriers into glucose and further utilized the glucose for their growth. Moreover, by crosslink with serum the STC carriers supported an even better cell production in the normal medium without adding fetal bovine serum, as well as a good extracellular virus production. The STC composite is therefore a promising alternative carrier for Vero cell culture.

Keywords Cell carrier · Carbohydrates · Chitosan · Vero cell

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Introduction

Cultured cells are widely used for the production of biological substances, in particular for biopharmaceuticals, such as vaccine and recombinant proteins (Han et al. 2006). A good growth of anchorage dependent mammalian cells are related to the material surface to which they are attached (Reid 1990) and an artificial (in vitro) environment for their subsequent growth. Microcarrier culture system has been developed to increase the area of cell growth and production but the microcarriers have to be kept in suspension (Nienow 2006). Fibra-Cel disks, another carrier type are predominantly used in perfusion processes because it enables sustained long-term periods of high-density growth in perfusion mode without danger of clogging (Merten et al. 2001; van der Loo et al. 2012). These two types of carriers have been widely used in commercial for vaccine production. Nevertheless, development of large-scale process conditions for the same cell line to produce different products is restricted to some factors, one major issue is the costs of carriers and the cost caused by contamination during cell culture operation (Zhang et al. 2010). Therefore, high price of these carriers and import dependence become the burden of manufacturing costs, particularly in Mainland China, Taiwan and Southeast region countries. Moreover, the use of serum in cell culture bears another issue of increasing difficulty of product purification due to the high protein content (Brunner et al. 2010).

Chitosan is a linear polysaccharide and nontoxic biopolymer. Currently, lots of studies have used chitosan as a matrix for cell attachment and growth in cell scaffold and tissue engineering, due to the properties of biocompatibility, biodegradability, ease to obtain and low cost (Gao et al. 2012). For bioreactor application, chitosan has been coated onto the surface of membranes for the purposes of antifouling, high flux and antimicrobial activities (Wang et al. 2010). Moreover, by mixing with other substances, such as fructose, aloe vera, other polysaccharides, proteins, cytokines or polyvinyl alcohol, the performance of chitosan-based polymers for cell growth can be improved (Kishimoto et al. 2009; Li et al. 2003; Lu 2009; Silva et al. 2005, 2010, 2013).

The aim of this work is to develop carbohydrate–chitosan composite carriers made of chitosan and various carbohydrates including glucose, sucrose and starch for the purpose of decreasing the cost of cell mass production from carrier itself. Glucose has a significant effect on cell growth and medium enrichment with glucose can enhance cell growth. Starch-based polymers are other potential biomaterials for applications to biomedical and environmental fields due to their biodegradability, low cost and renewability (Alberta Araújo et al. 2004; Lu 2009). We report an improvement in Vero cell growth using carbohydrate–chitosan composite carriers. At the same time, the starch–chitosan composite carriers crosslinked with serum were used to cultivate cells in a normal growth medium without serum addition, avoiding the difficulty of product purification in high serum protein content.

Materials and methods

Cells and virus

Vero cells [green monkey kidney cells, ATCC number: CRL-1587, purchased from Food Industry Research and Development Institute (Hsinchu City, Taiwan)] were routinely maintained in M199 medium (GIBCO, Life Technologies Corporation, Carlsbad, CA, USA) containing 10 % fetal bovine serum (FBS). Enterovirus 71 (EV71), a member of the enterovirus genus of the family of Picornaviruses, was used in this study. The virus EV71 was propagated in Vero cells using medium M199 with 2 % fetal bovine serum (FBS). Adsorption of EV71 to cells was carried out at a multiplicity of infection (MOI) of 1, at 37 °C for 1 h. The cells were then washed three times with phosphate-buffered saline (PBS) and incubated at 37 °C in M199 supplemented with 2 % FBS. When 80 % of the cells showed the typical enteroviral cytopathic effect (CPE), the infected cells were subjected to two freeze–thaw cycles for the virus harvest and stored at −80 °C for further use.

Preparation of carbohydrates–chitosan composite carriers

To prepare carbohydrates–chitosan beads, an amount of 3.0 g chitosan powder (molecular weight of 310 kDa, degree of deacetylation of 95 %, Kiotek Corporation, Hsinchu City, Taiwan) was dissolved in 100 ml of 1 % (v/v) acetic acid solution containing 10–30 % (w/v) glucose, sucrose and starch, respectively. To form beads, this solution was extruded through a syringe needle (25G) into a coagulant bath of 1 N sodium hydroxide solution (pH 11) containing 26 % (v/v) ethanol under stirring to form spherical uniform gel beads. These beads were then rinsed with distilled water until neutrality and then dried at 60 °C. The dried beads were autoclaved and then kept at room temperature for further use.

FT-IR spectral analysis

The chemical structure of the surface of carbohydrates–chitosan beads was analyzed by a FT-IR spectrometer (Thermo Spectra-Tech Co. HT32, Waltham, MA, USA) between 4000 and 500 cm^{-1} with a resolution of 4 cm^{-1}. These carbohydrates–chitosan beads were dried before use.

Vero culture on carbohydrates–chitosan composite carriers

An initial concentration of 5×10^4/mL Vero cells was seeded in a 50-ml centrifuge tube containing 0.15 g of dried carbohydrates–chitosan beads and then incubated at 37 °C with 5 % CO_2. For examining cell growth, one 50-mL centrifuge tube with beads was taken at each interval. The total beads were washed with 5 ml of PBS once and with 1 ml of a 0.25 % trypsin solution for 5 min at 37 °C to detach the cells from the surface of beads. After 4 ml of the growth

medium had been added, a hemacytometer was used to count the number of cells in each tube.

Glucose concentration in the medium was determined by DNS method. Briefly, 1.5 mL of 3,5-dinitrosalicylic acid (DNS) reagent was added to 0.5 mL of culture medium sample. The reacting solution was heated at 90 °C for 5 min to develop the red-brown color. After cooling to room temperature, the absorbance with a spectrophotometer at 540 nm was recorded. Glucose at concentrations of 0.1–1 g/L was prepared for standard curve.

Cross-linking of carbohydrates–chitosan composite carriers with serum

Dried carbohydrates–chitosan beads (0.15 g) were incubated with glutaraldehyde for 30 min and then washed with distilled water three times. Subsequently, the beads were incubated in fetal bovine serum (FBS) for 30 min and the treated beads were then washed with distilled water three times. For experiments, 5×10^4 cells/mL were seeded on the carriers. For chitosan composite carriers cross-linked with serum, M199 medium without serum addition was used for cell growth

Viral titration

Virus titers were determined from the median end-point of the tissue culture's infectious dose ($TCID_{50}$). Serial dilutions of virus (10^{-1}–10^{-8}) in M199 containing 2 % FBS were inoculated onto confluent Vero cell monolayers in 96-well plates and incubated for 4 days at 37 °C. After 4 days, the $TCID_{50}$ values were measured by counting the cytopathic effects in infected Vero cells. End-point titration was determined as the reciprocal of the lowest viral dilution that resulted in CPE in more than 50 % of cells in the 96 wells.

Statistics

All experiments were performed at least three times and standard errors of means (SEM) of all data were computed. Comparisons between the control and carbohydrates–chitosan composite carriers were performed by Student's paired t test. Tests were performed two-tailed and a value of $p < 0.05$ was considered as significant.

Results and discussion

Characteristics of carbohydrates–chitosan composite carriers

The sizes of carbohydrates–chitosan composite carriers of glucose–chitosan (GC), sucrose–chitosan (SC), starch–chitosan (STC) and chitosan control are shown in Fig. 1.

Among these carriers, STC carriers presented the biggest size of 0.3 cm diameter; the size of others was 0.2, 0.18 and 0.21 cm diameter for pure chitosan, GC, SC and STC carriers, respectively (Fig. 1a). After drying, the size of all carriers got reduced to at least half size of wet status (Fig. 1b). The dried carbohydrates–chitosan composite carriers, due to its hydrophilic nature, were able to absorb and reserve a certain amount of water when they were in the medium, to provide a growth environment. The water absorption ability reflects capability of the scaffold holding aqueous medium which is necessary for the cell growth (Luangbudnark et al. 2012). The water holding property of starch chitosan polymeric blend was superior to pure chitosan (Maitra and Singh 2014).

FT-IR spectral analysis

The IR spectra of chitosan, glucose, sucrose, starch, GC, SC, STC composite beads are shown in Fig. 2. The IR spectrum of glucose, sucrose and starch in Fig. 2a showed intense and characteristic bands in the regions between 3700 and 3000 cm^{-1} (OH stretching), 1849–1636 cm^{-1} (C=O stretching),

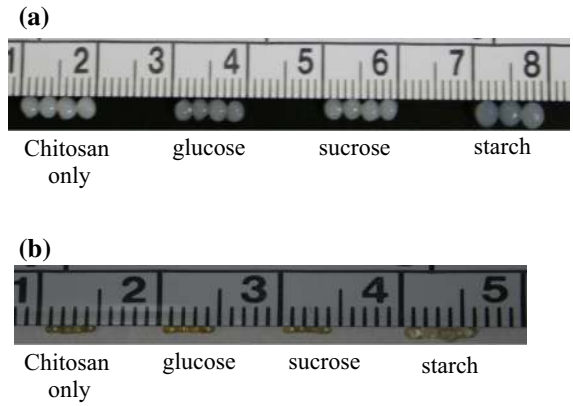

Fig. 1 Morphology of carbohydrates–chitosan composite and chitosan (control) carriers **a** wet carriers, **b** dried carrier

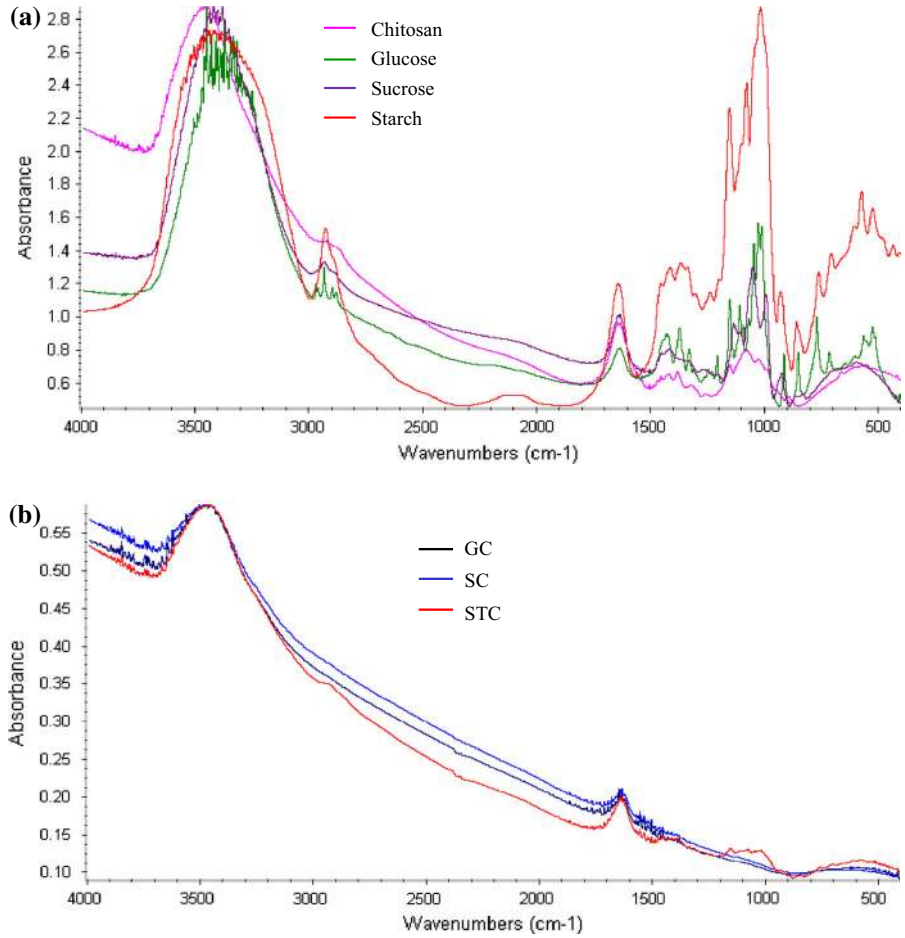

Fig. 2 FTIR spectrum **a** chitosan, glucose, sucrose and starch, **b** GC, SC and STC composite carriers

1526–1347 cm^{-1} (OCH and COH stretching), 1362–1191 cm^{-1} (CH and OH stretching), and 1191–995 cm^{-1} (CO and CC stretching). These results are found to be similar to previous reports (Adina et al. 2010; Huang et al. 2006; Medhat et al. 2006). The IR spectrum of chitosan, however, showed three characteristic peaks at 3455 cm^{-1} (OH stretching), 1646 cm^{-1} (NH2), and 1080 cm^{-1} (C O C stretching), similar to the previous report by Subramanian et al. (2014).

The results obtained for carbohydrates–chitosan composite carriers compared with data from pure chitosan, glucose, sucrose and starch, show that the regions of the characteristic bands at 1526–1347, 1362–1191, and 1191–995 cm^{-1} disappeared (Fig. 2b). Only two peaks remained in all composite beads and were somewhat different from each other. The 1634 cm^{-1} peak in glucose shifted to 1652 cm^{-1} in GC composite beads. The 1635 cm^{-1} peak in sucrose shifted to 1652 cm^{-1} in SC composite beads. The 1646 cm^{-1} peak in starch shifted to 1635 cm^{-1} in STC composite beads.

Vero cell growth on carbohydrates–chitosan composite carriers

The growth of Vero cells on the carbohydrates–chitosan composite carriers is shown in Fig. 3. These carbohydrates–chitosan composite carriers were translucent, except for STC composite carriers which were opaque. Vero cells adhered to the surfaces of chitosan control as well as GC and SC composite carriers for proliferation as anchored monolayers without any aggregate formation (Fig. 3a–c); however, cells formed clusters on STC composite carriers (Fig. 3d). After trypsinization, cells were easy to

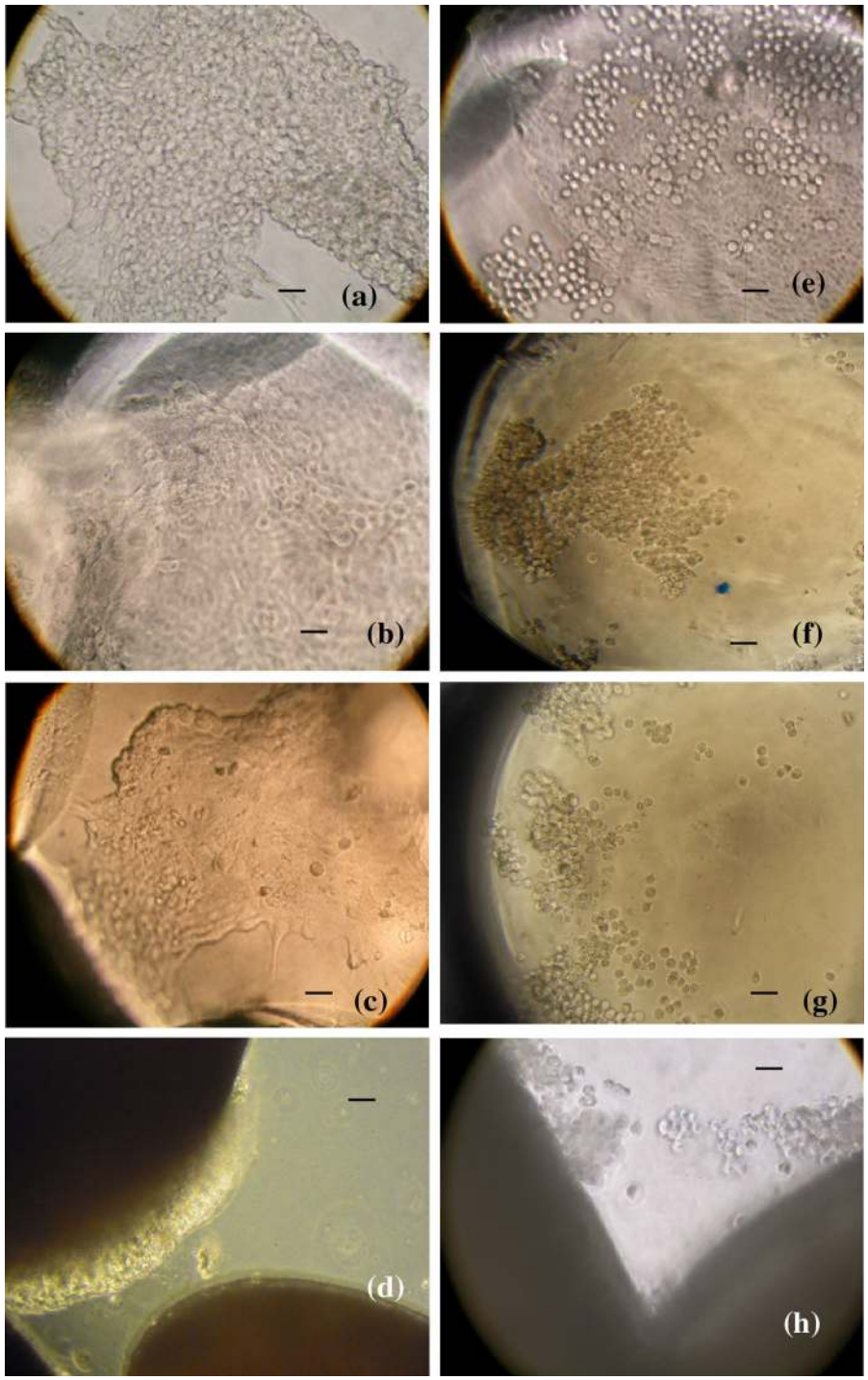

Fig. 3 Growth of Vero cells on **a** chitosan control, **b** glucose–chitosan, **c** sucrose–chitosan and **d** starch–chitosan composite carriers. After trypsinization, Vero cells easily detached from the carbohydrates–chitosan composite carriers of **e** chitosan control, **f** glucose–chitosan, **g** sucrose–chitosan and **h** starch–chitosan. The magnification was 40×. *Bar* 100 μm

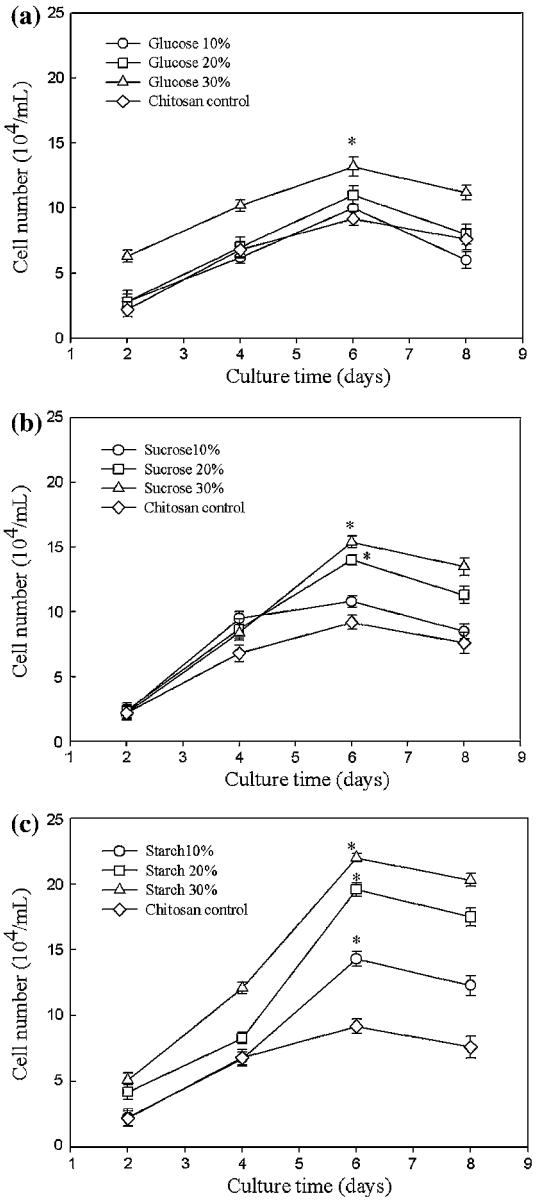

Fig. 4 Growth curves of Vero cells on **a** glucose–chitosan (GC), **b** sucrose–chitosan (SC) and **c** starch–chitosan (STC) composite carriers
* statistically different with respect to the control ($p < 0.05$)

detach from the surface of these composite carriers (Fig. 3e–h).

The cell growth peak on the carbohydrates–chitosan composite carriers was observed on Day 6 (Fig. 4). Vero cell production on these carbohydrates–chitosan composite carriers depended on the carbohydrate types and the content. Cell production increased in the following order: STC > SC > GC

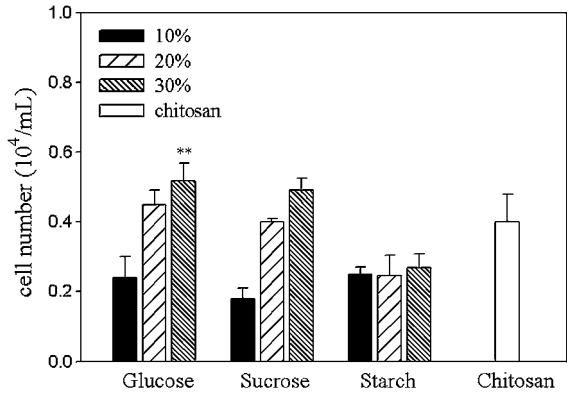

Fig. 5 Effect of cell attachment on the carbohydrates–chitosan composite carriers
** statistically different with respect to the control ($p < 0.01$)

and chitosan; moreover, high carbohydrate content in composite carriers actually increased cell production. The highest cell concentration of 2.02×10^5 cell/ml was achieved on 30 % STC composite carriers (Fig. 4c), which was twofold higher than the chitosan control and fourfold higher than the original amount of inoculum.

The efficiency of cell attachment on the carbohydrates–chitosan composite carriers was examined at 6 h after cell seeding. Figure 5 shows that all carbohydrates–chitosan composite carriers prepared were suitable for cell attachment and subsequent growth. However, the GC and SC composite carriers as well as pure chitosan carriers were more favorable for cell attachment than the STC carriers. An increase in the content of glucose or sucrose increased the cell attachment that was not observed in the cases of STC composite carriers.

Figure 6 shows the glucose concentration in the culture medium during the growth period of Vero cell on the carbohydrates chitosan composite carriers. Decreasing glucose concentration corresponded to cell growth which was observed in the cases of GC, ST and chitosan control carriers (Fig. 6a, b). However, cell growth was accompanied with an upward glucose concentration in the case of STC carriers; particularly in 30 % STC carriers a significantly increasing glucose concentration over 1 g/L was followed by a rapid cell growth (Figs. 4c, 6c). These results indicate that Vero cells were able to convert either sucrose or starch from the composite carriers into glucose and further utilized the glucose for their growth.

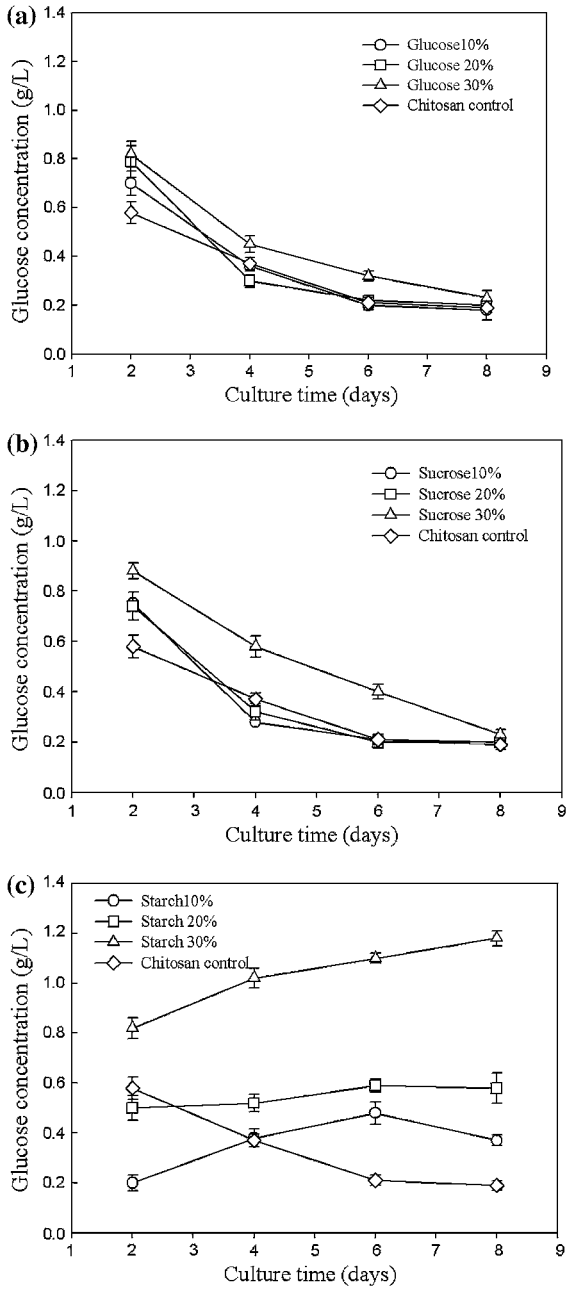

Fig. 6 Profile of glucose concentration during the growth period of Vero cells on **a** glucose–chitosan (GC), **b** sucrose–chitosan (SC) and **c** starch–chitosan (STC) composite carriers

Cell proliferation on cell carriers is dependent on three major factors. Firstly, cells must be able to attach on the surfaces of biomaterials; secondly, the surfaces of biomaterials have to provide optimum conditions for cellular migration and mitotic cell division, finally the nutrient, glucose in particular, has to be sufficient in the medium. The cell attachment on the surfaces of chitosan resulted from the electrostatic force between the cationic chitosan and negatively charged cell membrane (Baran et al. 2012). High polarity may result in strong cell attachment, large spreading area of cells and low cytocompatibility at the beginning of growth stage, leading to poor conditions of cellular migration and mitotic cell division for consecutive cell proliferation (Baran et al. 2012). As observed in the cases of GC, SC and pure chitosan carriers which are highly polar, there was stronger cell attachment and there were more attached cells on the surface of carriers in comparison to the STC carriers (Fig. 5); however, lower cell proliferation was obtained. In fact, a single GC or SC chitosan carrier with 0.13 cm^2 surface areas can provide at least 3×10^6 Vero cells to attach to it. It is obvious that other factors such as glucose concentration in the medium and/or the ability of carriers to provide cellular migration and mitotic cell division, affect cell growth. The results of lower cell proliferation on GC or SC chitosan carriers could be ascribed to low activity of cell division and migration in subsequent growth stage, rather than the size of carrier.

The presence of nonpolar starch in the carriers, significantly present in the composition at 30 wt%, generated low area of cell attachment at the initial stage and formation of cell clusters; therefore the consecutive cell proliferation was enhanced. Optimal cell spreading area on low cationic surface and short length of cells could induce an active cell migration and increased cell proliferation (Mattila and Lappalainen 2008).The study of chitosan conjugate membranes for osteoblast-like cells (SaOS-2) culture also indicated that the presence of low chitosan concentration or higher levels of fibroin and starch composition resulted in a significant decrease of cell attachment area followed by increased cytocompatibility and cell proliferation (Baran et al. 2012).

Additionally, glucose amount in growth medium also plays an important factor for cell growth. Higher glucose amount detected in the medium of GC and SC carriers contributed to a better cell growth in these carriers than in pure chitosan carrier. For Vero cell growth, control of glucose level at 1 g/L during the culture could maximize cell density (Trabelsi et al. 2006). This also explains the best growth of Vero cells on 30 wt% STC carriers, where glucose concentration

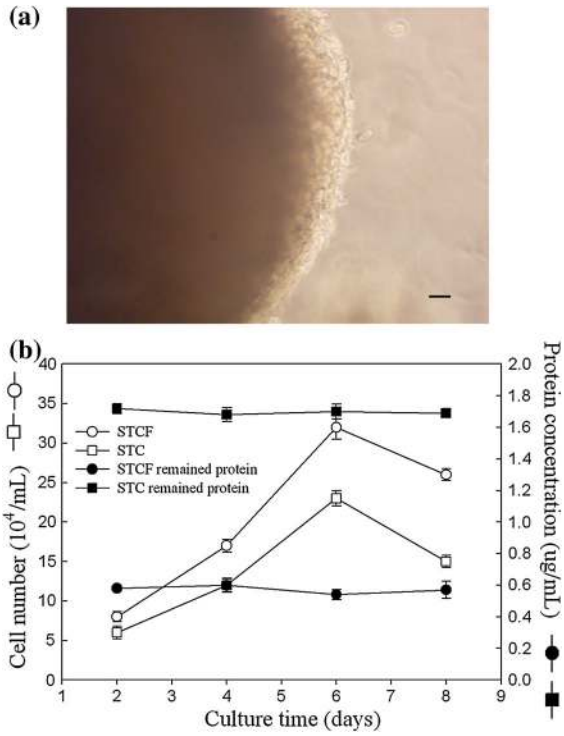

Fig. 7 **a** Growth of Vero cells on starch–chitosan composite cross-linked with serum. **b** The growth curves of Vero cells and the concentration of remaining protein in the medium during the growth period of Vero cells on starch–chitosan (STC) and starch–chitosan composite carriers cross-linked with serum (STCF). *Bar* 100 μm

was continuously maintained at about 1 g/L in the medium during culture period.

STC carrier crosslinking with serum for Vero cell growth

The 30 % STC carriers therefore were cross-linked with serum and used for Vero cell growth. Cell cluster appearance on STCF carriers was observed as on STC carriers (Fig. 7a). The growth profile of Vero cells observed in STC cross-linked with serum carriers (STCF) was higher than that obtained with the STC carrier (Fig. 7b). A maximum cell concentration of a 3.22×10^5 cell/mL was obtained, sixfold higher than the original inoculum. The content of proteins majorly from serum in the medium of STCF carriers was much lower than the protein contents in the medium of STC carriers during the period of cell growth, indicating

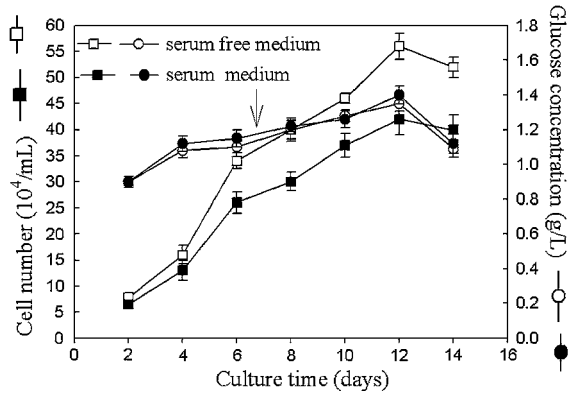

Fig. 8 Growth curves of Vero cells and the glucose concentration in the medium during the growth period of Vero cells on starch–chitosan cross-linked with serum (STCF) and STC composite carriers. Vero cells on STCF and STC carriers were provided with serum free medium and serum containing medium, respectively. New medium addition was performed on day 7(↓)

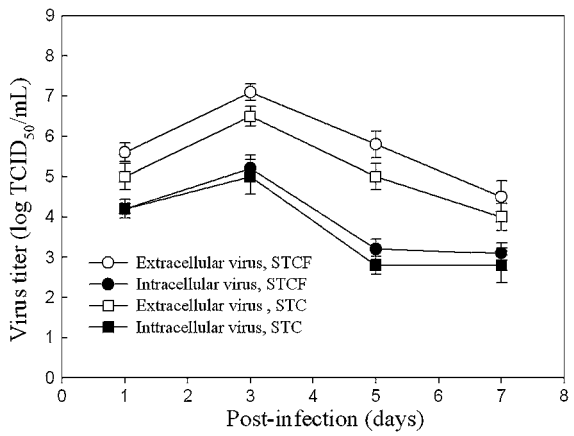

Fig. 9 Profile of EV71 production on STCF composite carriers

that cross-linked serum proteins did not significantly shed from STCF carriers.

The medium of Vero cell growth on the STC and STCF carrier was replaced at day 7 with the same, fresh medium. The growth of Vero cells continued on both carriers. The cell yield on STCF carrier was higher than on STC carrier, achieved the highest cell yield of 5.53×10^5 cell/ml on day 12, tenfold higher than the original inoculum (Fig. 8). Glucose in the medium was also continuously released into the medium with a level of 1–1.4 g/L until day 12.

EV71 growth on STCF carriers

The STCF and STC carriers were used to produce EV71 virus. When maximum cell growth was achieved on both types of carriers, cells were infected with EV71 at an MOI (multiplicities of infection) of 1. After three days post infection, almost 100 % of the cells were infected resulting in a maximum extracellular virus titer of 1.25×10^7 $TCID_{50}$/mL and 3.1×10^6 $TCID_{50}$/mL on STCF and STC carriers, respectively (Fig. 9). Productivity of the virus was superior on STCF without serum in the medium than on STC with serum-supplemented medium. EV71 produced by Vero cells generally stays inside of the cells and is referred as intracellular virus (Wu et al. 2004). Our results indicate that most of virus produced from either STCF or STC carriers was released into the medium, providing a benefit for virus harvest. The extracellular virus is directly collected from the culture supernatants; moreover, no serum protein was presented in the medium. Therefore, the cost of virus purification resulting from removal of serum protein and the infected cell lysates for intracellular virus harvest can be reduced.

Serum-free media have been well developed and commercialized; however, the price of serum-free medium is not cheap. Most serum-free formulae are designed for use in a microcarrier environment and cells have to be adapted to grow in such a medium (da Costa-Silva et al. 2012; Toriniwa and Komiya 2008). By cross-linking with serum, the STCF carriers, therefore, can reduce the costs from spending time and efforts adapting cells to a serum-free medium.

Conclusion

Both starch and chitosan are abundant naturally, cheap, non-toxic, biodegradable and antibacterial. The starch blending with chitosan composites have been developed for tissue engineering (Bacáková et al. 2014; Rodrigues et al. 2012) because of their good water absorption and film forming properties to support cell growth, attributing to the inter- and intramolecular hydrogen bonding that formed between amino groups and hydroxyl groups on the backbone of two components (Baran et al. 2004; Lu 2009). Additionally, the starch and chitosan based polymeric blends have been reported to possess antibacterial properties against Gram-negative pathogenic and nonpathogenic bacteria (Singh and Maitra 2015). Our 30 % STC composites provided not only a favorable environment but also a higher glucose level ~ 1 g/L in the medium during exponential growth phase for cell proliferation and further virus production. Furthermore, contamination from bacteria can be avoided during cell culture operation. For cost consideration, our STC composites can be promising candidates as cell carriers for cell production and virus production.

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