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Chemical Engineering Journal 135S (2008) S82–S88

Chemical Engineering Journal

www.elsevier.com/locate/cej

Attachment and detachment of living cells on modified microchannel surfaces in a microfluidic-based lab-on-a-chip system

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Abstract

The attachment/detachment of living cells on modified microfluidic channel surfaces has been investigated using a "lab-on-a-chip" system. Cell attachment was mediated using surface modifications of the microchannel based on three different reagents, namely, 3-aminopropytriethoxysilane (APTES), glutaraldehyde and collagen, whilst the detachment was carried out by flowing media through the microchannel at increasing flow rates. All three surface modification methods showed significant improvement for Chinese hamster ovary (CHO) cells' attachment, compared to that for an unmodified glass surface. The attachment/detachment of an additional four cell types, namely, T47D, U937, CaCo2 and NCTC 2544 cells, were also examined using the APTES modified channel surface. The observation of cell deformation suggested that the control of a shear stress within an optimal range enhances the cell adhesion to the surface. A theoretical model for fitting the measured detachment data is reported based on flow shear stress and the contribution from both surface adhesion bonds and hydrodynamic viscous stresses. It was demonstrated that the microfluidic system provided an easy and controllable approach to examine the attachment/detachment of a range of cells on different modified surfaces.

Keywords: Living cells; Microfluidics; Surface modification; Shear stress; Cell detachment

1. Introduction

The development of miniaturized "lab-on-a-chip" systems for chemical and/or biological applications has increasingly attracted interest over the last decade [1–5]. Such microfluidic-based microsystems represent the potential to "shrink" conventional bench chemical systems to the size of a few square centimetres with major advantages in terms of speed, performance, integration, portability, sample/solvent quantity, automation, hazard control and cost. These advantages are important for a variety of applications in analytical chemistry, biochemistry, clinical diagnosis, medical chemistry and industrial chemistry [6,7]. Consequently, numerous micrototal-analysis-systems (μ-TAS) and microreactor systems have been developed, and many more are currently under investigation [4].

For the study of biochemical or biomedical systems involving living cells, it is often a requirement that cells are delivered and remain at a desired location for examination and from where they can subsequently be removed. Our previous studies have demonstrated that microfluidics allows reagents and particles including cells to be manipulated and delivered to desired locations with programmable sequences within microchannel networks [5,6,8,9]. The location and then removal of cells from a given area generally involve an attachment/detachment process between cells and the substrate surface which can be mediated by channel geometry and flow rate (shear stress). The attachment of cells on a surface is normally performed by allowing cells to settle down without fluidic disturbance for a certain period of time. In some cases a chemical or biochemical modification of either cell surfaces or substrate surfaces can enhance the adhesion [10]. To remove cells from the substrate surface appropriate washing solutions are employed to break the bonds or cellular 'glue' that attaches the cells to the substrate and to each other by using proteolytic enzymes such as trypsin and dispase [11,12]. Whilst these methods are commonly used for batch or static operations, the shear stress caused by a fluid flowing along the substrate has been used as an effective approach to detach cells from the surface [13], which can be operated in either a continuous-flow or a paused-flow format [14] in a highly controllable manner [15,16]. The kinetics and mechanics of cell adhesion on substrate surfaces under shear stresses have been studied since the 1980s [17,18]. However, most of this work has been carried out from a mechanical engineering point of view. For instance, two commonly used models are based on either peeling-off-adhesive-tape or receptor–ligand-binding processes

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doi:10.1016/j.cej.2007.07.054

[19]. The mechanism, however, of attachment/detachment of living cells on a substrate surface represents a much more complicated system especially when the physical–biological interaction, involving hydrodynamics, adhesion strength and cell deformation is considered. More recently, research has been directed towards the interface of biology and material science for the study of interaction between cells and substrates under physiologically relevant shear stress conditions [20–23]. Furthermore, some dynamic surfaces using self-assembled monolyers have been developed allowing real-time control of the presentation of ligands in order to sequentially release and attach cells to a substrate [24,25].

In the present study, the attachment/detachment behaviour of living cells on modified microchannel surfaces has been studied by exploring the fluidic properties of a microfluidic-based "lab-on-a-chip" system. The microchannel surface was modified using three different surface modification reagents, namely, 3-aminopropytriethoxysilane (APTES), glutaraldehyde and collagen to enhance cell adhesion. The attachment/detachment of Chinese hamster ovary (CHO) cells to these modified surfaces were investigated. In addition, four other cell types, namely, T47D (breast tumour cells), U937 (human lymphoma cells), CaCo2 (human colon adenocarcinoma epithelial cells) and NCTC 2544 (keratinocytes cells), were also examined using a silanized channel surface. The deformation of cells under shear stress within microfluidic channels was also studied. A theoretical model for fitting the experimental data has been developed by taking account of shear stresses and the contribution from both surface adhesion bonds and cells deformability.

2. Experimental

2.1. Microfluidic chip fabrication

The microchip was fabricated according to published procedures [26,27] with minor adaptations. Briefly, the channel network was fabricated based on a photolithographic fabrication method. The channel network was designed using AutoCAD LT 2005 drawing software (Autodesk, Farnborough GU14 6FG, UK). A film negative of the desired final size was then prepared by a commercial photo mask manufacturer (J.D. Photo Tools, Oldham OL8 1EZ, UK) to form the optical mask. B-270 glass photolithographic plates (thickness of 3 mm) coated with a thin chromium metal mask layer plus an upper layer of positive photoresist, supplied by Telic (Telic Company, Valencia, CA 91355, USA), were used for channel network fabrication. With UV exposure, the pattern of interconnecting channels was transferred from the optical mask to the photoresist layer which was then developed and removed, together with the chromium layer, to reveal the channel areas of glass to be etched. The channels were etched by using a mixture of 1% (w/w) HF and 5% (w/w) NH_4F in water at 65 °C for 20 min, resulting in an etch channel network with a depth of 60 μm.

A base plate containing the etched channel network was sealed by bonding to an upper plate (also 3 mm thick) containing predrilled holes (diameter 1.5 mm) in order to link the ends of the channels with external tubing. The upper plate was aligned with the channel geometry and thermally bonded to the base plate by heating in a conventional furnace at a temperature of 575 °C for 3 h. Thermal bonding was aided by placing a 90 g block of stainless steel on the upper plate. After bonding, ETFE (ethylene tetrafluoroethylene) polymer tubing with an inner diameter of 250 μm was inserted into the drilled holes on the top plate and epoxy glue was used to secure the joint. On–off valves were connected to the other end of the tubing. The ETFE tubing (P/N. 1529) and on–off valves (P/N. P-782) were obtained from Upchurch (Upchurch Scientific Inc., Oak Harbor, WA, USA).

Fig. 1 shows the chip setup and configuration used, which consists of a double-T network with two inlet channels and two outlet channels. The geometry of the channel network is shown in the micrograph (Fig. 1b). The depth for all the channels was 60 μm.

2.2. Microchannel surface modification

The presence of surface modification to assist in the immobilisation of cells was located in a specific area in the microchannel

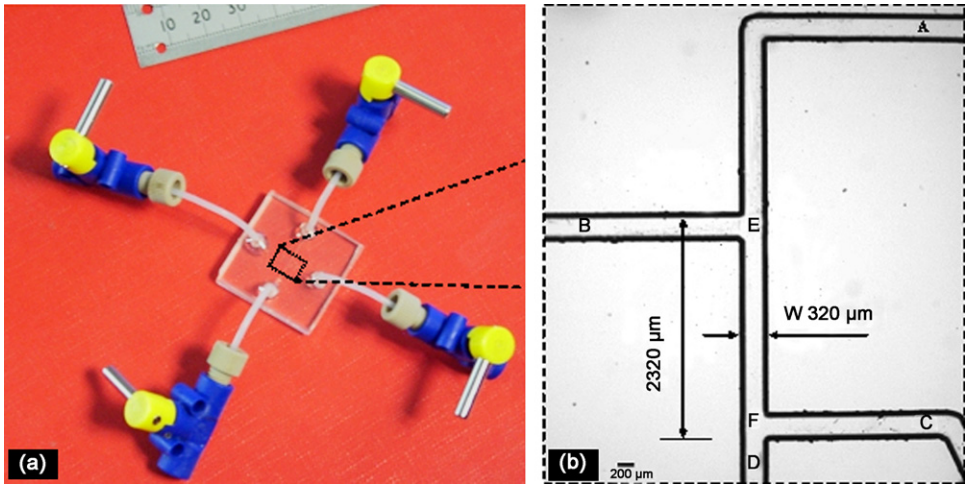

Fig. 1. (a) Glass chip with tubing and on–off valves and (b) outline of channel network.

between points E and F (Fig. 1b). Three modification agents were used, 3-aminopropytriethoxysilane (APTES), glutaraldehyde and collagen. In order to modify the selected channel area on the chip, the modification reagent was directed from B to C while channels A and D were closed. This flow pattern allows only the channel section between junctions E and F along the main channel to be modified.

2.2.1. Silane coating

To silanize the selected area on the microchannel surface, a solution of 10% (v/v) 3-aminopropyltriethoxysilane or APTS (99%, Sigma–Aldrich Company Ltd., Dorset SP8 4XT, UK) in ethanol was infused across the channel B–C at a flow rate of 10 μL min^{-1} for 30 min. The microchannels were then rinsed by continuously flowing 60% ethanol followed by DI water, both at a flow rate of 5 μL min^{-1} for 30 min. The channel network was then dried by blowing N_2 through.

2.2.2. Glutaraldehyde coating

Following the silanization step, a second layer of glutaraldehyde was coated which was obtained from Sigma–Aldrich as 50 wt.% solution in water. To perform the coating, 6% (v/v) glutaraldehyde solution in 0.01 M phosphate-buffered saline (PBS, with 0.0027 M potassium chloride, 0.137 M sodium chloride, pH 7.4, Fluka) was infused at a flow rate of 5 μL min^{-1} for 1 h across the selected microchannel section between junctions E and F along the main channel (Fig. 1b). The microchannels were then washed by continuously flowing 60% ethanol at a flow rate of 5 μL min^{-1} for 30 min. The channel network was then dried by blowing N_2 through.

2.2.3. Collagen coating

Following the silanization and glutaraldehyde coating steps, a collagen coating was performed on the top of the previous coatings. The agent was prepared by dissolving 1.0 mg collagen, type I from calf skin supplied by Sigma–Aldrich, in 1 mL acetic acid (0.1N) at room temperature, followed by adjusting pH to 8.3 with 0.1N NaOH. After the solution was filtered through a 0.2 μm filter, it was infused across the selected area within the microchannel network at a flow rate of 15 μL min^{-1} for 30 min. The channel was then washed by continuously flowing PBS buffer at a flow rate of 5 μL min^{-1} for 30 min. The channel network was then dried by blowing N_2 through.

2.3. Cell culture

CHO-K1 (Chinese Hamster Ovary, *Cricetulus griseus*) cells were supplied by ATCC/LGC Promochem (ATCC® No. CCL-61™, LGC Promochem, Middlesex TW11 0LY, UK). T47D, U937, CaCo2 and NCTC 2544 cells were obtained from the Medical Research Laboratory of the University of Hull. All cells were cultured in DMEM/F-12 medium (Cat. No. 21331-020, Invitrogen Ltd., Paisley PA4 9RF, UK) with a minor modification for each cell type according to the published procedures [15,28–31], respectively. A humidified incubator was used at 37 °C supplying 5% CO_2 in air. The concentration of cells used in this experiment was in the range of 7.5×10^6 cells mL^{-1}.

2.4. Instrumentation, image analysis and experimental procedures

Two KDS 200 syringe pumps (KD Scientific Inc., Holliston, MA 01746, USA) were used to deliver cells in suspension and carrier media. ETFE polymer tubing, on–off valves, and appropriate fittings and connectors were all obtained from Upchurch (Upchurch Scientific Inc., Oak Harbor, WA, USA). The viscosity of the media was measured using a modified Ubbelohde viscometer tube. All the measurements were carried out at room temperature.

An Axiovert S100 inverted microscope (Carl Zeiss, Hertfordshire AL7 1JQ, UK) using both transmission and fluorescent optics coupled with a monochrome CCD digital camera (C4742-95-12NRB, Hamamatsu Photonics, Hertfordshire AL7 1BW, UK) was used to obtain both conventional micrographs and digital videos of the microchip. AQM Hamamatsu ORCA I software (Kinetic Imaging, Nottingham NG8 6PE, UK) was used for image acquisition and analysis.

To deliver cells to the selectively modified area, channel surface between junctions E and F (Fig. 1b), cells in suspension were injected from inlet B to outlet C while channels A and D were closed. Following the infusion of cells into the chip all channels were closed in order to allow the cells settle down over a 30 min period without fluidic disturbance. To measure the cell detachment from the channel surface, the carrier media was introduced from channel A to D while channels B and C remained closed. The flow rate was incrementally increased, i.e., 1, 2, 5, 10 μL min^{-1}, and after each adjustment of the flow rate, an additional running period of 2 min was given to allow the chip to equilibrate at the programmed flow rate.

At each of the flow rates used two snap images covering the selected area were taken and subsequently analysed. The cell detachment was calculated as a percentage of cells removed from the surface as a result of the induced shear force. After a series images had been acquired, the cells were counted for the selected area on the channel surface. The extent of cell detachment from the channel surface was calculated as a percentage of the initial cell number (Eq. (1))

$$\text{detachment}\,(\%) = \frac{\text{initial cell number} - \text{remaining cell number}}{\text{initial cell number}} \times 100 \quad (1)$$

3. Results and discussion

3.1. Effects of surface modification on cell attachment/detachment under shear stress

Shear stresses are generally measured as the ratio of the shearing force to the area over which it acts. For the etched channels which have an approximately rectangular cross-section [27], the

Table 1
Summary of experimental conditions and corresponding shear stresses

Fluid viscosity, μ (dyn s cm^{-2})	0.01045
Channel depth, h (cm)	0.006
Channel width, w (cm)	0.032

Flow rate, Q (μL min^{-1})	Shear stress, τ (dyn cm^{-2})
0	0
1	0.9
5	4.5
10	9.1
20	18.1
60	54.4
90	81.6
110	99.8
150	136.1
190	172.4
230	208.6
290	263.1
350	317.5

wall shear stress under laminar flow conditions is calculated as [14,32,33]

$$\tau = \frac{6\mu Q}{wd^2} \quad (2)$$

where τ is the shear stress in dyn cm^{-2}, μ the fluid viscosity in dyn s cm^{-2} or P, Q the volumetric flow rate in cm^3 s^{-1} and w and d are the channel width and depth in cm, respectively. Table 1 summaries the experimental conditions and corresponding shear stresses calculated using Eq. (2).

Fig. 2 shows the detachment of CHO cells as a function of shear stress for an unmodified and three modified channel surfaces. The results showed that all the modified surface resulted in a significant retention of cells, compared to the unmodified surface. It can be seen that, in general, with an increase in shear stress, induced by increasing flow rate, more cells were detached from the channel surface. However, when the shear stress was increased above a certain point the detachment tended to reach a stable level indicating that the remaining cells attached to the surface in a firm manner. More details will be discussed in later sections in order to understand further about this observed trend. It was also found that surfaces modified by collagen provided the strongest attachment.

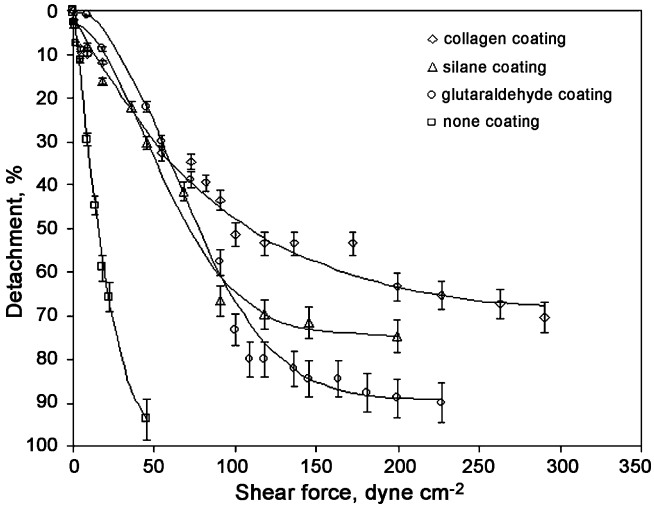

Fig. 2. Detachment percentage of CHO cells from the four types of modified microchannel surface as a function of shear stress. The solid curves are calculation results based on the theoretical model, and the error bars show a 95% confidence.

Generally, any forces between the cell surface and the channel surface can contribute to the attachment/detachment of cells, including hydrodynamic viscous forces, electrostatic affinity, chemical bonds and/or biological complex binding. In the silanization process with APTES, it has been reported that the chemical reaction between the glass surface and the silanization reagent allows amino groups to link by covalent bonds to the silicon atoms of the glass [10], and will leave the glass surface positively charged. Since the surface of cells is generally charged negatively, the electrostatic force can enhance the adhesion of cells to the positively charged channel surface.

Glutaraldehyde has been commonly used to fix cells whilst preserving their original shape and rendering them rigid [34]. This approach has been used notably for blood cells [35,36]. It has also been used as a pre-treatment for substrate surface to facilitate further modification [15]. It has been reported that the cell fixation process can result in an increase in cell's adhesion to the substrate [37]. In this study, glutaraldehyde was used to modify the channel instead of the cells' surface. The results (Fig. 2) confirm the enhancement of cell adhesion, although its mechanism remains unclear while some explanation has been proposed. Burks has found differences in zeta potentials of bacteria following glutaraldehyde treatment [35]. Brown et al. [37] found that glutaraldehyde caused yeast cells to become more hydrophobic. It is generally accepted however that glutaraldehyde can stiffen cells by cross-linking proteins and amino acids in the peptidoglycan layer [38].

Collagen is present as a major component of the extracellular matrix in many tissues and can influence cell proliferation, differentiation and migration [39]. Although the effects from collagen on cell behaviour are not fully understood, it is clear that it can be used to enhance the attachment contact or properties of cell with surface. Based on the biochemistry of collagen, the collagen monomer, tropocollagen, is a rigid rod-shaped molecule consisting of three polypeptide chains (α-chains) wound around each other to form a triple helix [40]. It is this particular structure that plays a big part interacting with the receptors on the cell surface, affecting cell surface glycosyltransferases, and influencing the mobility of proteins in the plane of the membrane [39].

To detach a cell from the surface by a fluid flow, a certain level of input fluid energy is required and distributed for two purposes (a) to overcome the hydrodynamic viscous resistance and (b) break adhesion bonds on the cell–substrate interface [13]. The hydrodynamic resistance can be generally treated in a similar way as for rigid, non-biological particles in fluidics. In contrast, the binding system on the cell–substrate interface is more complicated, as discussed above, which strongly depends on the cell's biological behaviour and activities in addition to their physical properties. Based on these assumptions, a simplified mathematical model has been used

$$P = P_{\max} - (P_{\max} - P_0)\, e^{-(\tau/a)^b} \quad (3)$$

Table 2
Four parameters obtained by best-fitting the experimental data into the model for CHO cells on four types of surfaces

	P_{max}	P_0	a	b
Collagen coating	70.17	1.44	89.13	1.05
Silane coating	74.83	2.96	68.87	1.80
Glutaraldehyde coating	89.24	0.12	85.05	2.01
No coating	101.25	0.02	21.02	1.25

where τ is the shear stress in dyn cm^{-2}, P_{max} the highest level of percentage detachment when high shear stresses are applied, P_0 the initial percentage detachment when the flow starts (being 0 in most cases). a provides a reference shear stress scale and b, the exponent reflects a threshold for cell removal which is a combination of contribution from cell–substrate affinity, cells deformability and hydrodynamic viscous affect. The four parameters have been obtained by best-fitting the experimental data, and are summarized in Table 2.

It is generally assumed that particles are "washed off" surfaces as fluid flow rates are increased because the hydrodynamic force can weaken the adhesion/attachment. This is indeed true for most of rigid mechanical particles. However, for a range of biological cells it has been reported that a threshold of fluid shear stress is required to sustain cell rolling on a substrate surface [41,42] and a maximum cell attachment is obtained. This has been mainly attributed to the chemical and/or biological bonds between the surfaces, and the deformation of cells which enlarges the cell–substrate contact area. In this study, an induction range was also observed, especially for cells with glutaraldehyde-coated surface (Fig. 2), indicating that cells were detached at a relatively low rate in the lower shear stress range. This effect is reflected on the b value in the model (Eq. (3)); a greater b value is an indication that a higher threshold is required.

3.2. Comparison of different cell types under shear stresses

It has been shown that all of the three surface modification agents investigated in this study can significantly enhance the attachment of CHO cells to the microchannel surface with collagen providing the strongest attachment. However as silanization proved to be the simplest surface modification method, in practice, it was used in this part of the work to study the attachment/detachment of a further four types of human cells, of general interest in biomedical research. The measured detachments of cells as a function of shear stress are depicted in Fig. 3. The calculated results based on the model (Eq. (3)) are also shown as the solid curves. By best-fitting into the experimental data the values of the four parameters in the model were determined and are summarised in Table 3.

It was observed that T47D cells from human breast tumours showed the strongest attachment to the channel surface whilst, in contrast, U937 human blood lymphoma cells appeared to be non-adherent with a lowest threshold. These results are in line with the general observation where breast cancer cells T47D are very adherent and tend to spread and attach to other tissues [43], whilst untreated monocytic U937 cells are unable to

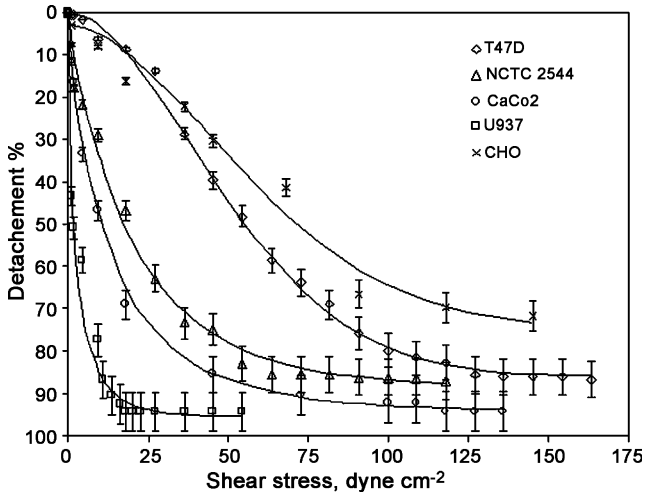

Fig. 3. Detachment percentage of five cell types on silanized microchannel surface as a function of shear stress. The solid curves are calculation results based on the theoretical model, and the error bars show a 95% confidence.

Table 3
Four parameters obtained by best-fitting the experimental data into the model for four cell types (in addition to CHO cell) on silanized channel surface

	P_{max}	P_0	a	b
T47D	86.00	0.01	59.64	1.81
NCTC 2544	88.66	0.09	21.43	0.88
CaCo2	94.01	0.00	14.65	0.77
U937	95.54	0.00	2.78	0.64

adhere to glass surfaces [44,45]. The human colon adenocarcinoma epithelial cells CaCo2 and skin-derived keratinocytes cells NCTC 2544 also showed some degree of adherence.

3.3. Cell deformation under shear stresses within microchannels

As discussed above, in addition to effects from cell–surface adhesion (chemically, or biologically) and hydrodynamic viscous factors, the deformation of cells under shear stresses is also important in the attachment/detachment process as it may lead to artifacts associated with cell differential. Fig. 4 shows a series of snap images for a single CHO cell within a microchannel under different conditions. It can be seen that the cell tended to spread on the surface after a period of settling down (Fig. 4b) and that the contact area with the surface increased. With a low shear stress applied (Fig. 4c), the contact area was enlarged further, and an increase in shear stress enhanced the cell deformation more significantly (Fig. 4d). After the flow was stopped the deformation recovered remarkably quickly (Fig. 4e).

Clearly, under shear stresses in a certain range, the cell appeared to be more "flat" to the surface aligning along in the direction of flow, decreasing the encountering cross-section area in both dimensions. Consequently, this deformation can reduce the fluid energy to the cell because a flattened cell will influence less disturbances to the flow and hence smaller shear stresses on the cell surface. In addition, the cell deformation can result in a larger contact area between the cell and the substrate surface.

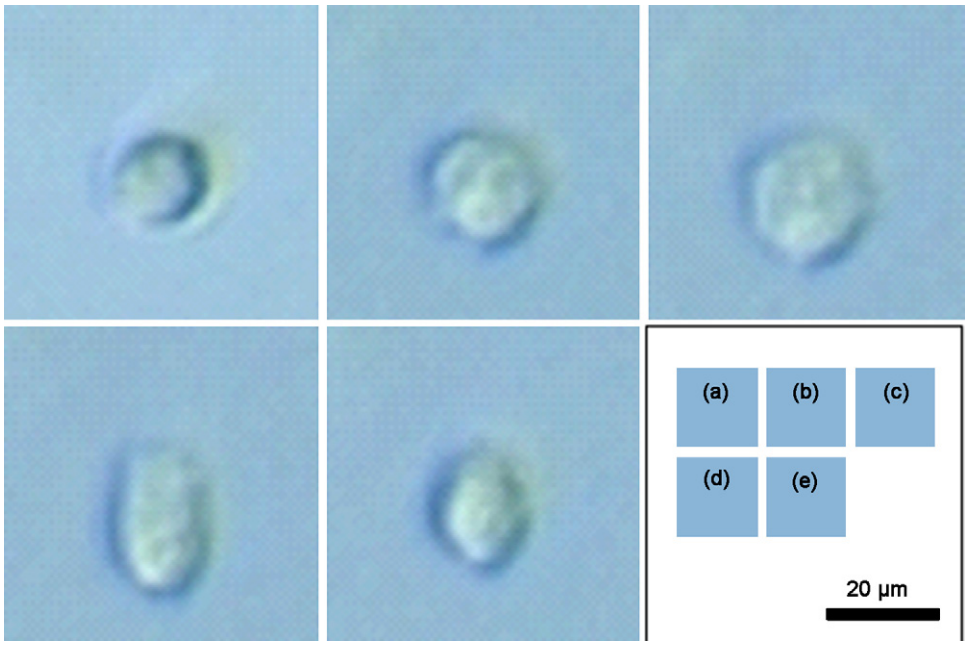

Fig. 4. Micrographs of a single CHO cell within a microchannel under different flow conditions. (a) The cell was delivered to position by injection flow, (b) followed by a 40 min settling, then subject to (c) a low shear stress of 11 dyn cm^{-2} and (d) a high shear stress of 274 dyn cm^{-2} by flowing in the media, and finally (e) the shear stress was released by stopping the flow.

It has been found that the contact area between the cell and the substrate could be nearly doubled under high wall shear stresses [13]. As a result, a higher shear stress was required to detach the cell from the surface. The observation of cell deformation under flow conditions can significantly contribute to the explanation for the existence of the threshold of shear stress to detach cells from surfaces. As can be seen from Fig. 5, that when encountering cross-section area with flow decreased, the contact area or adherent bonds (Fig. 5b) with substrate increased, so requiring more energy to detach the cell from the substrate surface.

It was also interesting to note that the cell recovered remarkably well from deformation when the shear stress was released which is probably due to the general surface effect where the cell tends to keep spherical due to its surface tension. It should be noted however that biochemical responses to shear stresses cannot be excluded. Frangos et al. reported that some cell functions may be modulated by membrane stresses induced by such artifacts as blood flow [46]. In addition, a recent investigation on the influence of hydrodynamic conditions on CHO cells activity in a microfluidic system found that a shear stress of 3.1 dyn cm^{-2} can cause a detectable fluorescence signal to be generated associated with intracellular Ca^{2+} transition within cells [47]. Clearly,

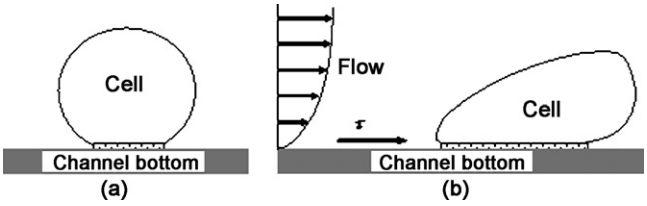

Fig. 5. Schematic of the cross-section of cells (a) attached on the surface of the microchannel bottom and (b) subject to a shear stress caused by the laminar flow.

to distinguish active actions from passive effects on the process of cell's deformation and its recovery, more investigation is required.

4. Conclusions

The behaviour of attachment/detachment of living cells on modified microfluidic channel surfaces has been studied in a "lab-on-a-chip" system for a range of cells with surfaces modified using different methods. The attachment was performed by modification of the microchannel surface using three different surface modification reagents, namely, 3-aminopropytriethoxysilane (APTES), glutaraldehyde and collagen. It was found that surfaces modified by collagen provided the strongest attachment for CHO cells whilst the silanization was the simplest method to use practically. The detachment of other four types of cells, namely, T47D, U937, CaCo2 and NCTC 2544 cells, were also examined on a silanized channel surface. The observation of cell deformation suggested that the application of a shear stress in a certain range could enhance the cell adhesion to the surface mainly due to the enlargement of contact area between cells and the microchannel surface. A theoretical model for fitting the measured detachment data has been developed by taking account of shear stresses and the contribution from both surface adhesion bonds and hydrodynamic viscous stresses. It was demonstrated that the microfluidic system provided an easy and controllable approach to examine the attachment/detachment of a range of cells on different surfaces.

Acknowledgements

We thank Ms. Huguette Savoie of the Department of Chemistry at the University of Hull for help with the cell culture.

The Medical Research Laboratory of the University of Hull is acknowledged for providing the cells.

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