Cellular responses to chitosan *in vitro*: The importance of deacetylation

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Chitin and chitosan (a deacetylated derivative of chitin) have been proposed for biomedical applications because of their biocompatibility and abundance in nature. We have investigated the effect of the percentage of deacetylation (%DD) of chitosan on biocompatibility from two sources, shrimp and cuttle fish, with two cell lines, L929 and BHK21(C13). The difference in %DD for each source was approximately 10% in the range of 76–90%. Biocompatibility was investigated for: (1) cell adherence and growth on the chitosan samples as substrate; (2) the effect of extract media on 2d and 7d growth; and (3) the presence of an inhibition zone. The results were similar for both cell lines. The chitosan samples were air-dried on to tissue culture-grade petri dishes to provide a substrate for the adherent-cell cultures. The higher %DD substrates from each source supported attachment of the cells, while the lower %DD did not. Cells cultured in medium conditioned by each substrate (i.e. extract medium) displayed an initial difference in growth which was abrogated in cultures incubated for 7 days. No inhibition zone was apparent. However, after 7 days, some cells were noted migrating on to the low %DD substrate disks. The morphology of these cells was changed with the presence of pseudopodia being apparent. Thus, especially with regard to attachment the %DD has a very important effect on the biocompatibility of the chitosan and should be monitored carefully.

1. Introduction

Chitin is one of the most abundant polysaccharides found in nature. This fibrous material is found in the exoskeletons of crustaceans and insects, in fungal cell walls and plankton. It is often considered a cellulose derivative. Chemical structures of cellulose, chitin and chitosan are shown in Fig. 1. Chitin is a linear polymer of repeating N-acetylglucosamine residues, it is not found alone and usually forms a part of very complex systems with other sugars, proteins, minerals and polyphenols in varying proportions. Chitin is normally insoluble in common solvents. Controlled deacetylation to produce derivatives with approximately 50% free amine can be used to produce water-soluble chitin. The chitin derivatives that contain more than 50% free amine in the structure are called chitosan; the percentage of deacetylation (%DD) determines the properties of these polymers. Chitosan is soluble in organic acids and dilute mineral acids. It can be fabricated in many forms such as membranes, sponges and beads. It is noted that the fully acetylated or deacetylated polymers are not naturally occurring [1].

Chitin and chitosan have been proposed for several biomedical applications because of their excellent biocompatibility and abundance. Chitin has been found to have an acceleratory effect on the wound healing process [2, 3]. Chitosan has been proposed for use as a homeostatic agent [4], and chitosan-bonded hydroxyapatite bone-filling paste has been proposed as a bone substitute [5].

The quantity of the N-acetyl group in chitin and chitosan molecules has influence on properties such as solubility, viscosity and crystallinity. Importantly, the prospective use of a biomaterial often depends upon the ability of the construction material either to support or reject cell attachment. In order to design an appropriate application for each material a study of the effect of % deacetylation of chitosan membranes on mammalian cell responses would be of interest. This paper reports results of the response of L929 and BHK21(C13) cells to chitosan extracted from shrimp shells and from cuttle fish with various percentages of deacetylation.

2. Materials and methods

2.1. Preparation of chitosan

Chitosan powders were extracted from two different sources; (a) from shrimp shell: encoded COM1 and COM2; (b) from cuttle fish: encoded CF1 and CF2.

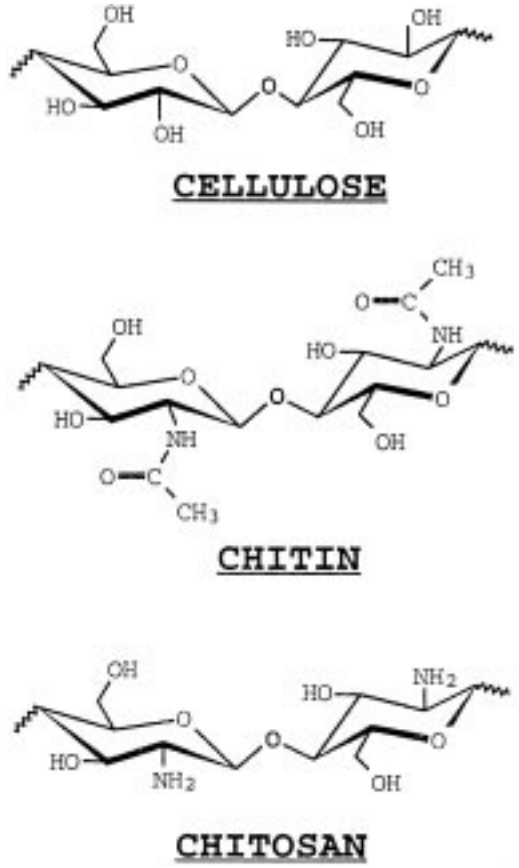

Figure 1 Chemical structures of cellulose, chitin and chitosan.

Chitosan solutions were prepared by dissolving the powders in 1% (v/v) acetic acid to make saturated solutions. The percentage of deacetylation of chitosan (%DD) was determined by the titration method with sodium hydroxide [6].

2.2. Cell lines and culture

Two cell lines were used in the assays: L929, mouse, connective tissue, fibroblast-like cells; and, BHK21(C13), baby hamster kidney cells. The growth medium used for all cells was Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS) and penicillin (100 U/ml) and streptomycin (100 μg/ml) (Gibco, Life Technologies, Paisley, Scotland, UK). All cells were cultured in 50 mm petri dishes (Nunc) with 4 ml medium at 37 °C in 5% CO_2. For adherence studies the cells were seeded on chitosan-coated dishes at the following densities: BHK21(C13) at 1×10^5/dish; and L929 at 2×10^5/dish. The seeding densities for the assays which require cell growth in extract medium and the inhibition zone assay were: BHK21(C13) at 1×10^4/dish and L929 at 2×10^4/dish.

2.3. Cytotoxicity assays

2.3.1. Direct contact assay

This assay conforms to ASTM Standard F813–83 (Reapproved 1992). Biocompatibility in these assays is defined as the ability to support cell adherence and growth. The experimental materials were used as substrates for cell adhesion and control dishes were uncoated tissue culture-grade plastic. Petri dishes (50 mm) were coated with 1.5 ml of the chitosan solutions and allowed to dry for 48–72 h in a laminar flow hood with the lids off and the blower on. All chitosan-coated dishes were washed twice with phosphate-buffered saline (PBS), pH 7.4. The cells were seeded and incubated for 48 h. Cell adhesion was assessed by microscopic examination. The attached cells were removed from the substrates with 0.06% trypsin (Sigma Chemical Co., Singapore) and counted with a haemocytometer. Cell viability was determined with 0.1% Trypan Blue (Sigma Chemical Co., Singapore). In the case of non-adherence, the condition of the cells was observed and noted; and for the determination of cell viability the growth medium with suspended cells was centrifuged at 1000 rpm for 5 min and the cells were resuspended in 1 ml of 0.1% Trypan Blue. Cell counts and viability were measured.

The biocompatibility of the materials as determined by the direct contact assay was confirmed with the inhibition zone assay. For this assay, a 100 μl drop of chitosan solution was placed in the middle of the 50 mm petri dishes and dried as for the ''substrate'' dishes. The cells were seeded on to the dish where adherence, mobility and the occurrence of an inhibition zone were assessed microscopically at days 2 and 7.

2.3.2. Indirect contact assay

The production of extracts complied with the ASTM Standard F619–79 (Reapproved 1991). Tissue culture dishes were coated with 1.5 ml of the chitosan solutions and dried as above. Complete growth medium was then added to the dishes at a concentration of 1 ml/2 cm^2 and incubated without shaking, for 2 days at 37 °C to produce the resultant conditioned media or ''extract medium''. All media contain phenol red, no change of color was noted indicating that the dried substrate had no pH effect on the medium. Control extract medium was obtained from uncoated petri dishes treated in the same manner. The effect of chitosan extracts on cells was investigated for both cell attachment and cell growth. For cell attachment, cells were seeded on 50 mm petri dishes in extract media and incubated for two days. The medium was removed and following a rinse with PBS the attached cells were detached with 0.06% trypsin and counted with a haemocytometer. For the cell growth assay in extract medium, the cells were seeded in growth medium to avoid any possible attachment interference from the extract medium and incubated for two days. The medium was then changed to extract media and the cells were incubated for a further five days. The number of cells in each dish was determined.

2.4. Statistical analysis

All data were analyzed by ANOVA with the Genstat® 5 release 3.2 Statistical System for Windows ™ [7].

3. Results

3.1. Deacetylation

Per cent deacetylation of the chitosan samples is shown in Table I. COM2 and CF2 were approximately 10% more deacetylated than COM1 and CF1, respectively.

TABLE I Characteristics of chitosan samples

Samples	Source	Concentration (w/v)	Ash content (%)	Deacetylation (%)
COM1	Shrimp shell	1.6%	<1	76
COM2	Shrimp shell	1.6%	<1	86
CF1	Cuttle fish	1.5%	<1	81
CF2	Cuttle fish	1.5%	<1	90

3.2. Cell adhesion and growth on substrates

The effect of chitosan substrates on cell adhesion was similar for both cell lines measured after 48 h in culture. The L929 and BHK21(C13) cells were able to adhere to, flatten on to, and grow on the COM2 and CF2 substrates. However, none of the cells, L929 or BHK21(C13), were able to adhere to COM1 or CF1. The resultant non-adherent cells were between 95–100% viable but the total cell number was similar to the seeding density. The results indicate no growth of the cells on the COM1- and CF1-coated dishes. All unattached cells showed a repeated tendency to form spheroids of live cells which excluded Trypan Blue.

The relationship of attachment and growth of the cells with the %DD of the substrate followed a general trend with the higher deacetylated substrates supporting attachment and subsequent growth of the cells whereas their counterparts did not. This difference was significant ($p < 0.05$) between CF1 and CF2 for both cell lines and between COM1 and COM2 for L929 cells. These results are presented graphically in Fig. 2.

3.3. Adhesion and two day growth in extract media

Both cell lines were able to adhere to the plastic tissue culture dishes when seeded in the four extract media and incubated for two days. Interestingly, the adherence and growth of all cells was greater in COM1 and CF1 than in the COM2 and CF2 extract media, respectively. The amount of growth followed the general trend with the higher deacetylated the chitosan substrates, the lower the growth of the cells. This difference was significant ($p < 0.05$) between COM1 and COM2 for both cell lines and between CF1 and CF2 for BHK cells. These results are illustrated in Fig. 3.

3.4. Seven day growth in extract media

The effect of extract medium on long-term growth was assessed over a period of seven days. All the cell lines grew equally well in the CF1 and CF2 extract media. However, there was a significant difference in the growth of BHK21(C13) cells in the COM1 and COM2 extract media ($p < 0.05$), with cell growth reduced in COM2 extract medium; no difference was demonstrated with the L929 cells. The effect of extract medium on cell growth is shown in Fig. 4.

3.5. Direct contact assay

No inhibition zone was present around any of the four substrate disks for the L929 and BHK21(C13) cells. In the dishes containing the COM1 and CF1 discs, all the cells consistently grew up to the edge of the drops and lined up parallel to it, but did not go on to the disk (Fig. 5a). After seven days in culture when the area surrounding the disks was overgrown, a few cells could be seen migrating on to the discs with extended pseudopodia (Fig. 5b, c). In contrast, in the dishes with COM2 and CF2 disks, L929 and BHK21(C13) cells attached well to the disks and grew as colonies. There was no lining up at the edges of these disks; the cells attached to the disks without pseudopodia, this was taken as an indication of attachment rather than migration (Fig. 5d, e). These observations are consistent with the

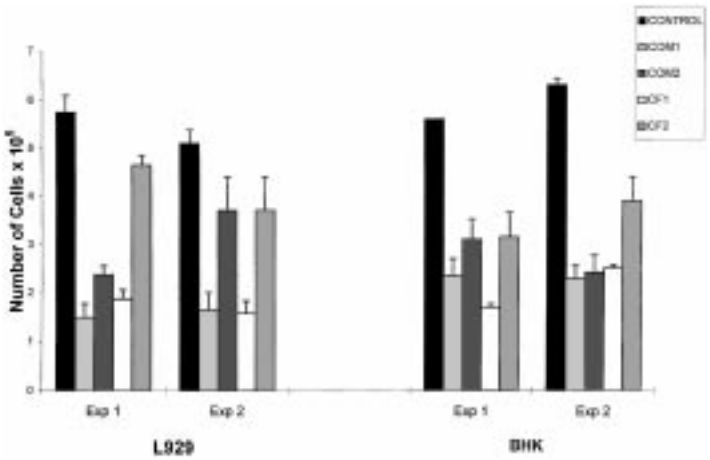

Figure 2 Cell adherence and growth. The number of cells harvested after 48 h incubation from the CF1-coated plates were significantly lower than those from the CF2-coated plates for both the L929 and BHK21(13) cells ($p < 0.03$). The number of L929 cells harvested from COM1 was significantly lower ($p < 0.01$) than COM2; and although the number of BHK21(C13) cells harvested from the COM1 substrate was lower than COM2, following the trend, the difference was not significant.

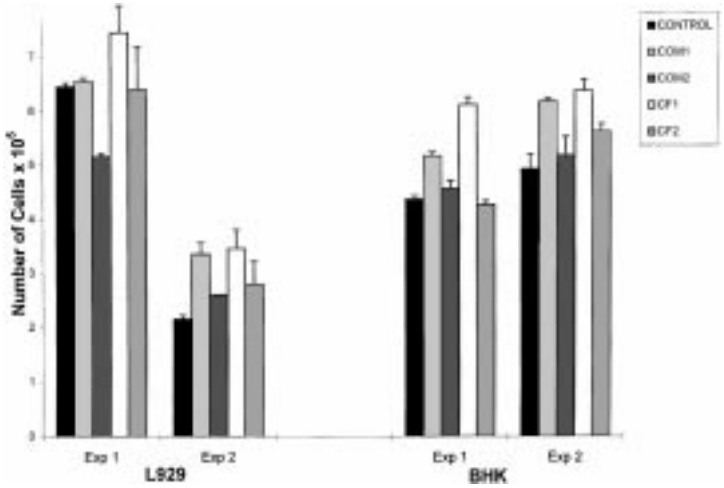

Figure 3 Adherence and two-day growth in ''extract'' medium. Both L929 and BHK21(C13) cell lines illustrated a significant difference in growth between COM1 and COM2 ($p = 0.003, p = 0.006$; respectively). Additionally, BHK21(C13) cells also demonstrated a significant difference of growth in CF1 and CF2 extract media ($p < 0.05$).

above results of cell adhesion on chitosan-coated dishes.

4. Discussion

The findings here show that the chemical composition of the chitosan, regardless of the source of the chitosan (either from shrimp or cuttle fish), has important effects on the biocompatibility of the dried substrates. The two cell lines investigated reacted similarly to the substrates and their extracts. The more highly deacetylated chitosans, COM2 and CF2, supported attachment of the cells; while the lower %DD substrates, COM1 and CF1, did not. Adherent cells in culture do not adhere directly to the culture vessel, but bind to an extracellular matrix (ECM) which in turn must adhere to the culture vessel surface. This ECM can be produced by the cells themselves or, alternatively, the culture surface can be coated with a suitable biomatrix to which the cells bind [9]. It is well known that cells carry a negative surface charge at physiological pH. The difference in cell adhesion to the chitosan samples can be explained by the fact that COM2 and CF2, have more active free amino groups in their molecules than their counterparts. The higher %DD of chitosan, the higher amount of free amino group (-NH_2), which in turn, can become protonated to form a cationic amine group (-NH_3^+) producing a positively charged surface; and it is this which allows interactions between the cells and the culture surface. This chemical characteristic present in chitosan has also been reported to have the capability of protein and enzyme fixation [10, 11]. Therefore, chitosan with the higher extent of deacetylation facilitates cell adhesion more efficiently than that with lower %DD. A novel finding of this study is the demonstration that as little as 10% difference in the extent of deacetylation of chitosan samples has a significant influence on the adhesion to these substrates by cells in culture.

It is of interest to note the COM2 and CF2 extract media, in both the two day cultures and the seven day cultures, supported less growth than their counterparts. The deacetylation process removes proteins as well as the N-acetyl groups from the chitosan. According to the chemistry of chitin and chitosan, there are two possible explanations for these results. First, COM2 and CF2 were subjected to more extensive deacetylation than COM1 and CF1 resulting in less protein bound to chitosan and more amino groups for protonation. Cations, particularly

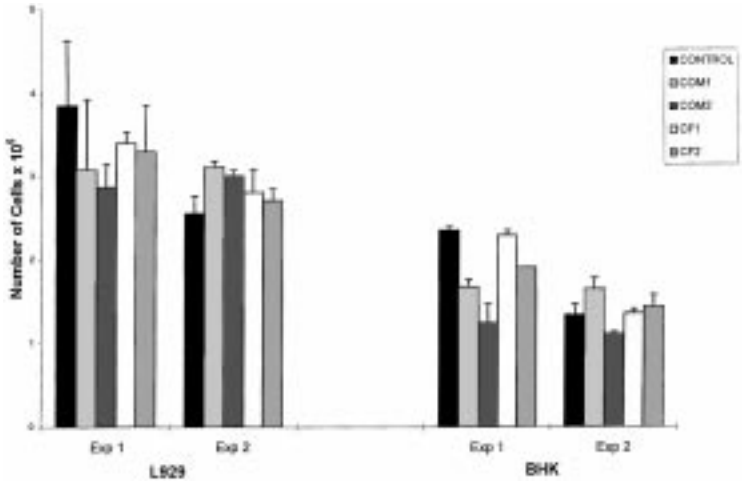

Figure 4 Seven days growth in ''extract'' medium. No difference in culture growth was demonstrated for L929 cells grown in either COM1 or COM2 extract medium, nor between CF1 and CF2 extract media. BHK21(13) growth was significantly less for cells grown in extract medium from COM2 than COM1 ($p < 0.05$); no significant difference in growth was shown between CF1 and CF2 media.

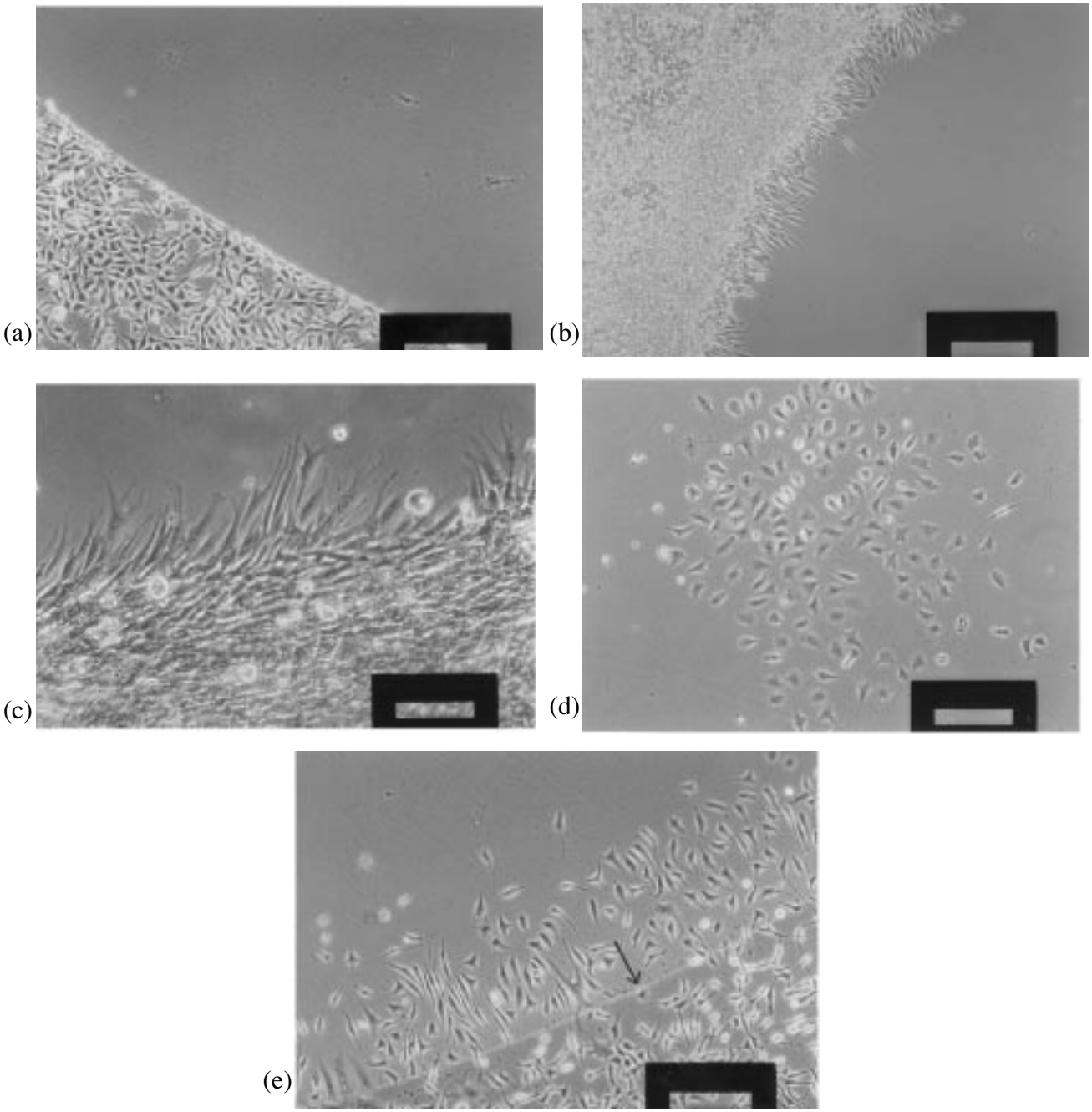

Figure 5 Inhibition zone assay. All cells were seeded in a petri dish with a disk of substrate located in the center. (a) L929 cells on tissue culture plastic (bottom) and the COM1 disk (top) after two days incubation. (b) L929 cells and the CF1 disk after seven days incubation, note pseudopodia and apparent migration on to the disk. (c) BHK21(13)cells and the COM1 disk after seven days in culture with pseudopodia and apparent migration. (d) L929 cells forming a colony on the COM2 disk. (e) L929 cells at the periphery of the COM2 disk (arrow), cells adhere equally well on the disk as on the tissue culture plastic [a and b: Bar = 200 μm; c–e: Bar = 100 μm].

Ca^{2+} and Mg^{2+} in FCS and DMEM, which mediate cell adhesion may be bound to the chitosan membrane during the extraction. Thus, necessary attachment factors would be partially depleted in the extract media. Poorer cell growth would be expected in extract medium containing lower concentrations of Ca^{2+} and Mg^{2+}. After seven days this difference was less than at two days, and was only significant for COM1 and COM2 with BHK cells. An alternative explanation is that non-specific proteins or amino acids possibly leached from the chitosan to the extract medium play a role in cell adhesion and cell growth in culture.

The inhibition zone test demonstrated that the cells could grow either on or up to the edge of the chitosan disks giving no support for the presence of an inhibition zone proximal to the substrates. Just as in the attachment experiments, initially the cells were able to attach to the COM2 and CF2, but not to the COM1 and CF1 substrates. However, after seven days incubation the cells in the overgrown cultures with the COM1 and CF1 substrates appeared to be migrating on to the disks. The morphology of these cells, because of the presence of pseudopodia-like extensions, differ from that of cells attached to the more highly deacetylated substrates, COM2 and CF2. While it appears that the cells did not go onto the COM1 and CF1 substrate disks until the cultures

were overgrown, another possible explanation is that after seven days in a liquid environment the substrates were more amenable to cell attachment and growth for an unknown reason. The actual mechanism of this cell attachment is unknown, but because of the importance of keeping the surfaces of some biomaterials free from adherent cells this phenomenon requires further investigation.

In summary, the high %DD substrates COM2 and CF2 were able to support attachment and growth of the cells. The cells were unable to attach to the lower deacetylated substrates COM1 and CF1, and hence these adherent-dependent cells were unable to grow. The initial growth of cells cultured in COM2 or CF2 extract media was reduced compared to COM1 or CF1 extract media; after seven days incubation this difference was reduced. No inhibition zone was present giving an indication that although the cells were unable to attach to the COM1 and CF1 substrates this was not as a result of cytotoxicity. The degree of deacetylation has been shown to be important to the biocompatibility of chitosan.

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