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Study on the effects of nylon–chitosan-blended membranes on the spheroid-forming activity of human melanocytes

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Abstract

Though reported limitedly in tissue engineering, modification of cellular functions can be achieved by culturing them into multicellular spheroids. We have shown melanocytes form spheroids on chitosan surface. However, how biomaterials promote spheroid formation has never been systemically investigated. In this work, nylon, which inhibits melanocyte spheroid formation, and chitosan, which promotes melanocyte spheroid formation, are used to prepare nylon/chitosan-blended membranes. Membranes composed of pure nylon, pure chitosan and various ratios of nylon and chitosan are employed to examine their effects on spheroid formation. Melanocytes show better adhesion to nylon membranes than that to chitosan membranes. In blended membranes, as more nylon is incorporated, cell adhesion increases and the trend for spheroid formation decreases. Melanocytes can only form spheroids on membranes with poorer cell adhesion. Examining the surface of the blended membranes shows phase separation of nylon and chitosan. As nylon content increases, the nylon phase on the membrane surface increases and thereby enhances cell adhesion. The opposite trend for cell adhesion and spheroid formation substantiates our hypothesis of spheroid formation on biomaterials: a balance between cell–substrate interaction and cell–cell interaction. The decrease in cell–substrate interaction tilts the balance to a state more favorable for spheroid formation. Our work can serve as a model to investigate the relative strengths of cell–cell and cell–substrate interactions and also pave way to design blended membranes with desired physical properties while preserving the spheroid-forming activity.

Keywords: Melanocyte; Chitosan; Nylon; Blend; Spheroid; Vitiligo

1. Introduction

Vitiligo, characterized by depigmented patches on the body surface, is a disorder resulting from loss of functional melanocytes in the epidermis [1,2]. The conventional non-invasive treatments of vitiligo include topical medication and irradiation using various light sources [1–3]. In cases recalcitrant to conventional treatments, autologous melanocyte transplantation by use of cultured melanocyte suspension has become popular recently [1,4–7]. This method has drawbacks including cell damage in cell preparation and transportation, difficult manipulation and disappointing engraftment rates for lesions on acral parts and periorificial areas [1,7]. To improve the above-mentioned drawbacks, the concept of using tissue-engineered "cellular path" for melanocyte transplantation was proposed in our previous reports [8,9]. In brief, the cellular patch is composed of two layers: a melanocyte layer on top of a supporting biomaterial membrane layer.

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During transplantation, the patch is used to cover the dermabraded vitiliginous lesion in an up-side-down orientation.

In the development of cellular patch for melanocyte transplantation, a unique phenomenon of human melanocytes was revealed on membranes composed of pure chitosan: formation of multicellular melanocyte spheroids [8,9]. We have shown that culturing melanocytes into spheroids on chitosan provides a survival advantage in stringent conditions [9], and thereby can potentially enhance the successful repigmentation rates of melanocyte transplantation.

Though reported limitedly in the field tissue engineering, culturing cells into dense aggregations or multicellular spheroids has been reported in several types of cells using a variety of biomaterials in specified conditions [10–13]. The function, growth and differentiation of cells can be differed via enhanced intercellular contact in the aggregated state. For example, the ability of producing albumin is increased in hepatocyte spheroids [12]. Aggregation also regulates cellular functions by increasing dopamine secretion in PC12 cells [14]. Neuron stem cells can retain their stemness only when they are cultured into neurospheres [10,11].

Up to date, the effect of blended membranes composed of polymers with and without spheroid-forming activity on the spheroid formation of cells has not been systemically investigated. Study on the effects of blended membranes on the cell aggregations may shed light on the mechanism of multicellular self-assembly on biomaterials. Also, this will pave way to design a blended membrane with desired physical properties while preserving the ability to facilitate spheroid formation of cultured cells. In this work, membranes composed of pure chitosan which promotes spheroid formation, pure nylon (nylon-66) which lacks spheroid-forming activity, and various proportions of each polymer are examined.

2. Materials and methods

2.1. Membrane preparation and characterization

Nylon (Nylon-66, DuPont Zytel 101, Mn = 87,000 g/mole), chitosan (Sigma C-3646, Mn = 810,000 g/mole, degree of deacetylation = 85%), and nylon/chitosan-blended membranes were cast from formic acid solutions. The membranes were prepared by the evaporation of solvent from the polymer solution as previously described [15]. Samples were designated as NC25, NC50, and NC75, labels indicating that the weight ratios of chitosan were 25%, 50%, and 75%, respectively, in the blended membranes.

The morphology of the membrane was examined using a scanning electron microscope (SEM). The dried sample was sputtered with gold in a vacuum and viewed using a SEM (Hitachi S-2600H).

Tensile testing was performed on water-swollen copolymer films as previously described with modification [16]. The prepared membranes were 15–300 μm thick (dumbbell-shaped specimens, width D 5 mm). Tensile tests were performed in 3-fold at room temperature with universal tensile testing machine (RTA-1T, ORIENTEN TENSIKON) operated at a cross-head speed of 2 mm/min, using an extensimeter, a 1 kg pre-load, and a grip-to-grip separation of 30 mm. The specimen elongation was derived from the extensimeter separation (10 mm).

For determination of the transparency, the prepared membranes, 35 mm in diameter, were placed in 6-welled tissue culture polystyrene plates (TCPS) (Costar, USA). The 6-welled TCPS was placed on a green plank with white checkers. The transparency of prepared membranes was photographed by a digital camera. In addition, the transparency was also quantified by determination of the light transmittance of the membrane by a home-built device. In brief, the initial illumination from a white light source was measured by a digital lux meter (MLM-1010, minipa®). Then a prepared membrane was inserted between the light source and the digital lux meter and the illumination was measured again. Transmittance was defined as the ratio of the detected illumination after insertion of the prepared membrane to that without insertion of the prepared membrane.

2.2. Cell culture, cell adhesion, cell growth and cell morphology

Human melanocytes were provided by cell bank of Department of Dermatology, National Taiwan University Hospital. The study protocol was approved by our Institutional Review Board. We conformed to the Helsinki Declaration with respect to human subjects in biomedical research. Human melanocytes were cultured using modified melanocyte medium (MMM) as previously described [8]. The flask was incubated at 37 °C in an atmosphere of 95% air/5% CO_2. After 17–22 days of 1–2 subcultures, the number of melanocytes increased 50- to 100-fold, with a doubling time of 2.6–3.7 days. The cells were then detached by trypsinization and spun down before the following experiments.

For determination of the cell adhesion and cell growth, the prepared membranes, 15 mm in diameter, were placed in 24-welled TCPS (Costar, USA). A Teflon ring 15 mm in diameter was placed on each of the tested membranes in the wells to prevent them from floating. Membranes and Teflon rings were sterilized in 70% alcohol overnight and rinsed extensively with phosphate buffered saline (PBS), followed by treatment under ultraviolet light overnight. Subsequently, 1 mL MMM of cell suspension at a density of 4×10^4 cells/mL was added to each well and maintained in a humidified atmosphere with 5% CO_2 at 37 °C.

For the measurement of cell adhesion, cells were washed with PBS to remove non-adherent cells, and the number of attached cells was determined 4 h after cell seeding by MTT assay [8,17]. The 200 μL MTT (Sigma) solution was added to each well. After 3 h incubation at 37 °C, the supernatant was discarded and dimethyl sulfoxide (DMSO) of 200 μL was added to dissolve the formazan crystals. The dissolvable solution was jogged homogeneously about 15 min by a shaker. The solution of each well was transferred to an eppendorf and was centrifuged at 1500 r.p.m. for 1 min to remove any possible melanocytes contaminated in the solution. The optical density of the formazan solution was read on an enzyme-linked immunosorbent assay (ELISA) plate reader (ELx 800, BIOTEK) at 570 nm. The absorbance was proportional to the number of cells attached to the membrane surface. To examine cell growth, the cell number on the membrane was determined 7 days after seeding by MTT assay also.

For morphological observations, the cells adhering to the membranes were washed by PBS after 4 h, 1 day, 3, 5 and 7 days of incubation and then fixed with 2.5% glutaraldehyde in PBS for 30 min at 4 °C. After thorough washing with PBS, the cells were dehydrated by graded ethanol changes and then critical point dried. The samples were then gold sputtered in vacuum and examined by SEM (Hitachi S-2600H).

3. Results

3.1. The tensile strength of nylon/chitosan-blended membranes

The tensile strength of prepared membranes was given in Fig. 1. In this study, the mean tensile strengths of chitosan and nylon membranes were 5.57 ± 0.33 and 5.57 ± 0.49 MPa, respectively. The increase in tensile strength

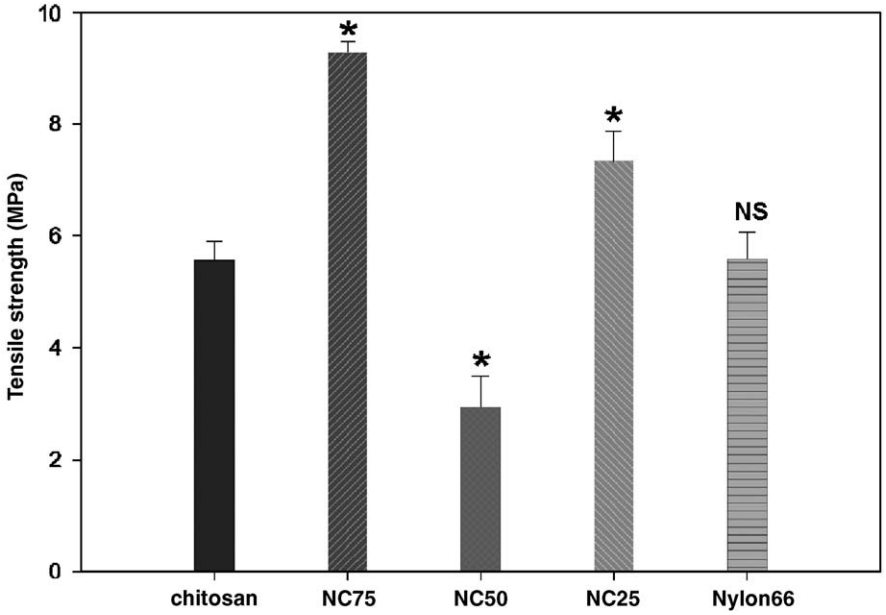


Fig. 1. The tensile strengths of the prepared membranes. The tensile strengths are not proportional to the content of nylon in the blended membranes. Blended membranes containing 25% nylon (NC75) have the highest tensile strength. Errors bars reflect standard deviations calculated from three independent experiments. Student's t test is performed against chitosan group for comparison (*$p<0.05$; NS: non-significant).

was not correlated with the increasing nylon content. The tensile strength of NC50 membrane (2.94 ± 0.55 MPa) was significantly lower than that of pure chitosan and pure nylon membranes ($p<0.05$), while the tensile strength of NC25 membrane (7.35 ± 0.48 MPa) was slightly higher than that of pure chitosan and nylon membrane ($p=0.04$ and 0.06). Clearly, NC75 membrane had the highest tensile strength (9.27 ± 0.21 MPa) in this study and was significantly higher than other prepared membranes ($p<0.05$). Compared with pure chitosan membranes, blending with 25% of nylon increased the tensile strength to approximately 1.7-fold in NC75 membrane.

3.2. The transparency of nylon/chitosan-blended membranes

During the melanocyte culture and transplantation, transparent membranes enable observation of the cell behavior, healing process and signs of possible infection. Therefore, transparency of prepared membranes is of significance in terms of clinical applications [9].

The result is shown in Fig. 2. The white checkers could be clearly observed through the empty TCPS due to the nature of its transparency (Fig. 2(a)). In the experimental group, the green plank and the white checkers could be easily observed through chitosan and NC75 membranes, indicating that chitosan blended with 25% of nylon could still preserve its transparency. On the other hand, as more nylon was incorporated into chitosan, the membranes become progressively opaque and the white checkers could only be hardly traced and the color of the green plank could not be observed through NC50, NC25 and nylon membranes, indicating that these samples were not transparent. Similarly, the detailed morphologies of melanocytes in culture could only be clearly observed via inverse phase contrast microscope in the groups cultured on chitosan and NC75 membranes (data not shown). Quantitatively, a similar decreasing trend of transmittance was observed as more nylon was incorporated into the blended membranes (Fig. 2(b)).

3.3. Characterization of membranes

The scanning electron micrographs of prepared membranes are shown in Fig. 3. Pure chitosan membrane displayed a flat dense surface (Fig. 3(a)) and pure nylon membrane had a particulate morphology (Fig. 3(e)). Membrane structures can be regulated by a variety of methods, one of which is to use blended polymers instead of single polymers. When nylon was incorporated, the flat dense surface of the chitosan membrane became progressively undulating as the nylon content increased. This result is in accordance with our previously report [15]. In the case of NC75, evenly scattered round plaque-like structures could be observed (Fig. 3(b)). As the content of nylon increased further, these round plaque-like structures increased in size, from around 5 μm in diameter in NC75 membrane (Fig. 3(b)) to more than 50 μm in diameter in NC50 membranes (Fig. 3(c)). As we have shown previously [15], due to the immiscibility of the two polymers, the phase separation of nylon phase may contribute to the round plaque-like structures in the blended membranes.

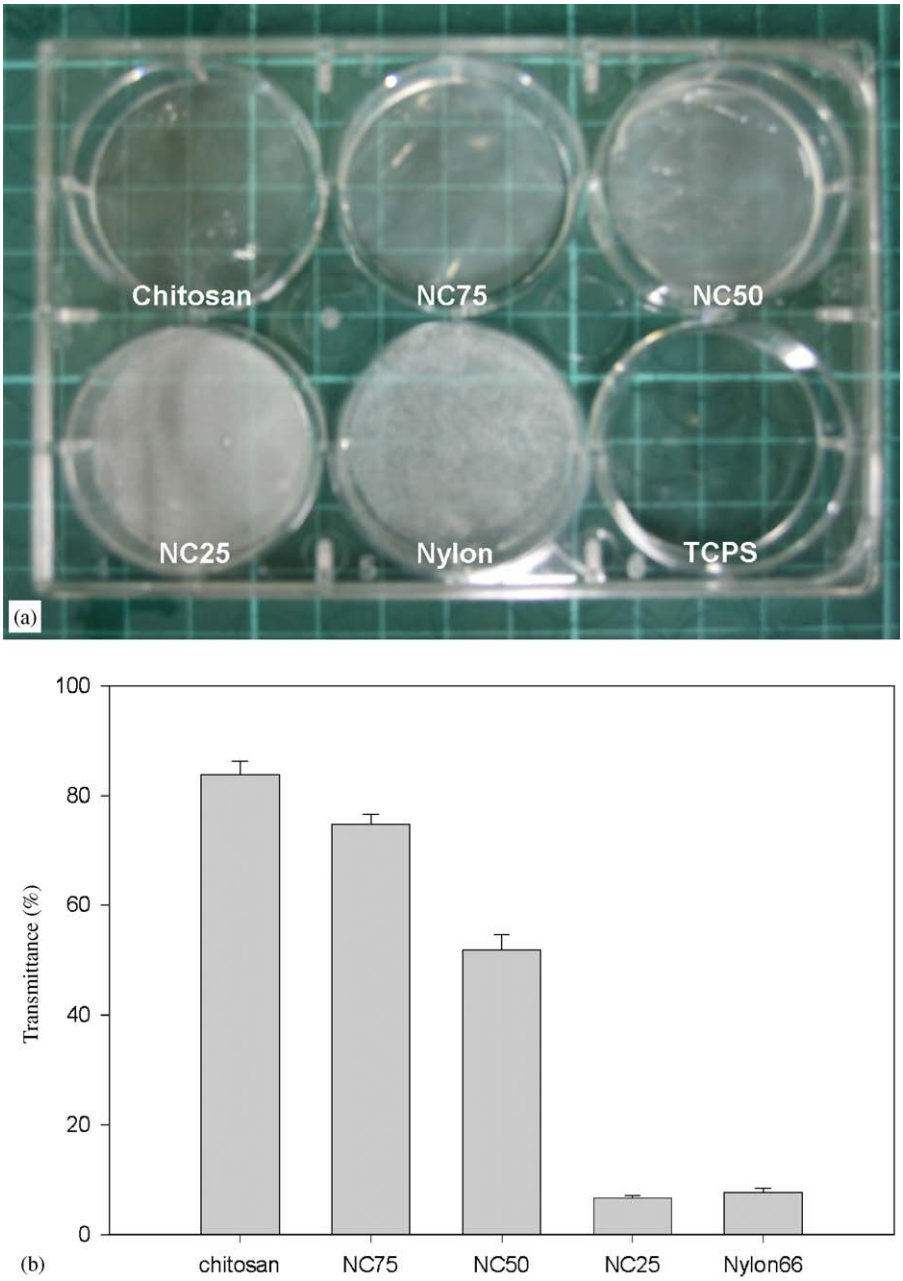


Fig. 2. The transparency and transmittance of the prepared membranes. (a) The white checkers can be easily traced through chitosan and NC75 membranes. As more nylon is blended into chitosan, the prepared membranes become progressively opaque. Nylon membrane itself is also opaque. (b) The transmittance was progressively decreased as more nylon was incorporated into the blended membranes.

3.4. Cell morphology

The cell morphologies 4 h to 7 days after seeding are shown in Fig. 4. In comparison with cells on pure nylon membranes that are of multiple dendrites (Fig. 4(e1)), melanocytes on chitosan and NC75 were mostly bipolar with short dendrites and a great proportion of cells still preserved a round morphology (Fig. 4(a1 and b1)). As nylon content increases, cell adhesion and spreading were enhanced indicated by the trend of increasing number and length of dendrites of melanocytes (Fig. (4c1, d1 and e1)). The findings suggest that the cell adhesion to the blended membrane is enhanced by the increasing nylon content in the membrane.

After 1 day in culture, the morphologies of melanocytes on all prepared membranes all showed a highly dendritic appearance with multiple cell processes (Fig. 4(a2, b2, c2, d2 and e2)). Flat dendritic piling-up cells can be focally observed on chitosan (Fig. 4(a2)), NC75 (Fig. 4(b2)) and NC50 (Fig. 4(c2)) membranes. However, no melanocyte spheroid was observed on all prepared membranes at the time point.

After 3 days in culture, melanocytes aggregated and grew into compact round spheroids on chitosan and NC75

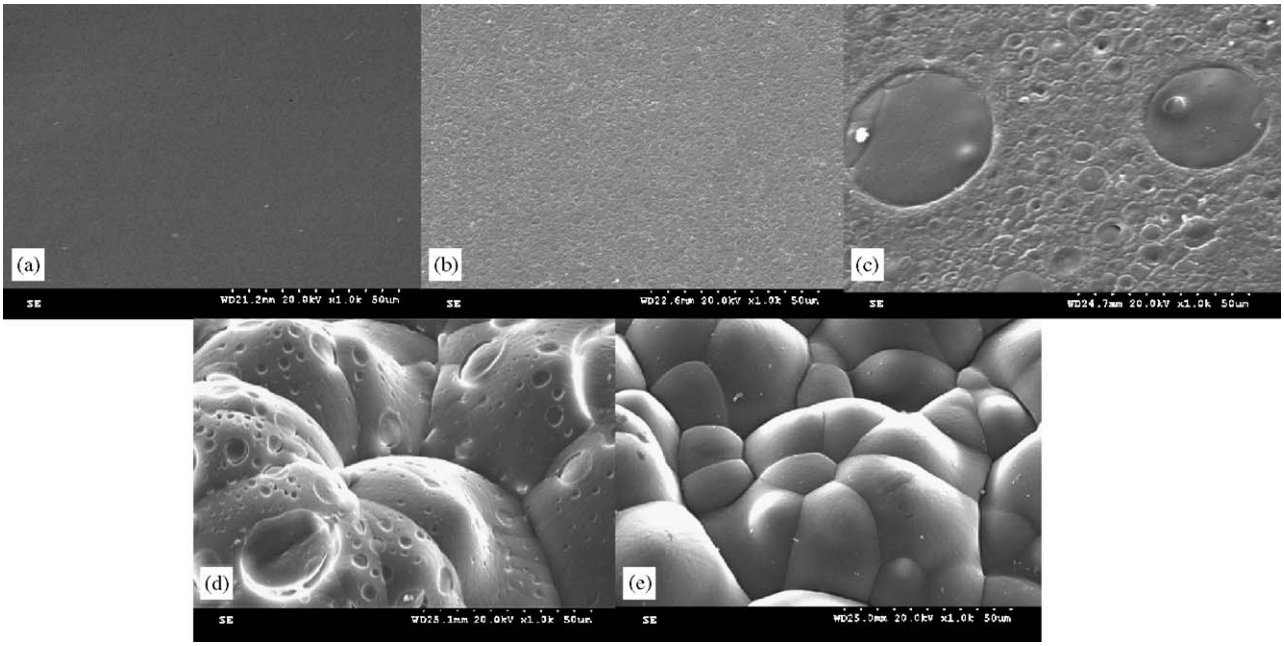


Fig. 3. The scanning electron micrographs of the top surface of prepared membranes. (a) chitosan, (b) NC75, (c) NC50, (d) NC25 and (e) nylon. The surface becomes progressively undulating as more nylon is incorporated into chitosan (Magnification $\times 1000$).

membranes (Fig. 4(a3 and b3)). On the contraty, although initial flat dendritic piling-up cells could be observed on NC50 membrane after 1 day in culture (Fig. 4(c2)), they failed to grow into compact spheroids (Fig. 4(c3)). In the groups of NC25 and nylon, cells still preserved a flat dendritic morphology (Fig. 4(d3 and e3)).

Maintained in culture for 5 days, more and larger multicellular spheroids developed on chitosan and NC75 membranes (Fig. 4(a4 and b4)). Between spheroids, dendritic melanocytes were still present. On the contrary, though focal flat aggregations of cells could be found on NC50, NC25, and nylon membranes (Fig. 4(c4, d4 and e4)), no melanocyte spheroid was observed. Melanocytes remained flat and dendritic on these membranes.

When the cells were maintained on the prepared membranes for 2 more days, multicellular melanocyte spheroids could only be observed on chitosan and NC75 membranes also (Fig. 4(a5 and b5)). Cells remained a monolayered dendritic morphology on NC50, NC25, and nylon membranes (Fig. 4(c5, d5 and e5)).

3.5. Cell adhesion and cell growth

Fig. 5 shows the MTT conversion of cells attached to the prepared membranes 4 h after cell seeding. Chitosan and NC75 membranes had lower MTT conversion. This indicated that melanocytes had a poorer adhesion to the chitosan and NC75 membranes, which was consistent with the results of scanning electron micrographs (Fig. 4(a1 and b1)) showing that melanocytes still preserved a round or bipolar morphology on chitosan and NC75 membranes 4 h after seeding. As the nylon content further increased (NC50, NC25 and nylon), the cell adhesion was enhanced (Fig. 5). The results were in accordance with the cell morphologies 4 h after seeding, showing that the increased nylon content enhanced the melanocyte adhesion.

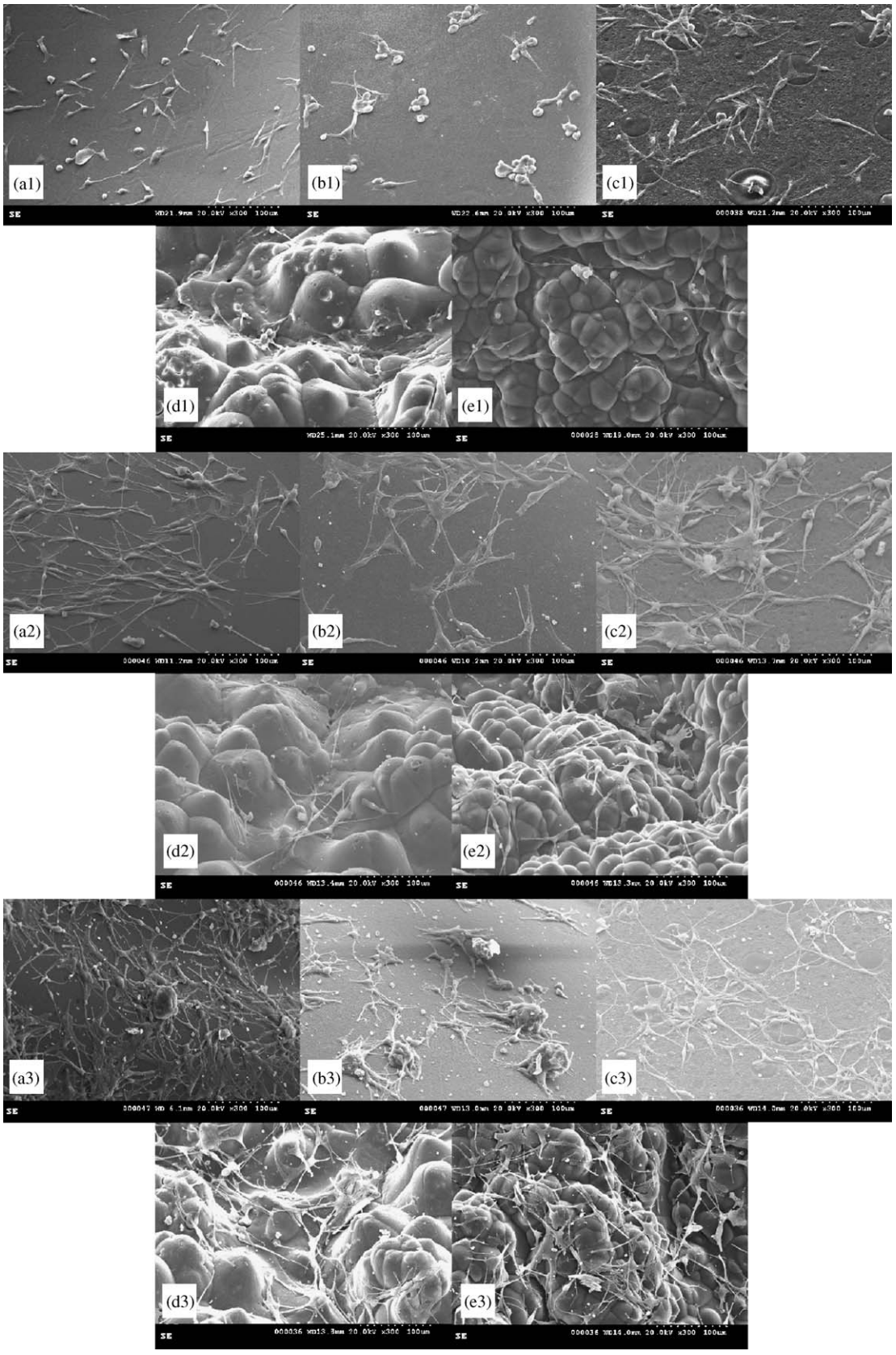
The result of MTT assay after 7 days in culture is shown in Fig. 6. In contrast to cell adhesion, the trend for cell growth was not correlated with the increasing nylon content. Cell growth was enhanced when a small amount of nylon was blended into chitosan (NC75) and became highest when an equal amount of nylon was incorporated (NC50). When more nylon was incorporated into chitosan (NC25), cell growth was reduced.

4. Discussion

Chitosan has been widely used for tissue engineering [18–21]. However, chitosan has a relatively low mechanical strength for clinical use. To enhance the mechanical properties of chitosan, a number of methods can be employed. For example, the mechanical strength of chitosan can be increased by cross-linking [22]. However, the cross-linked membrane may become brittle and decrease in its strain. Another example is that chitosan can be prepared directly from crystalline chitin [23]. Though the tensile strength is increased, the chitosan membrane obtained from such preparations is limited in size and may not be suitable for melanocyte transplantation.

Another widely used method to modify the properties of biomaterials is via blending with other polymers [15,24–29]. It has been shown that the mechanical properties as well as the biological effects on cells will be varied in the blended membranes [15,25,26]. Interestingly, when a second polymer is incorporated to prepare a blended membrane,

we have shown that the change in the biological effects on cells, such as cell proliferation and cytokine secretion of cells, is not linearly correlated with the increasing content of the newly incorporated biomaterial [15]. As to melanocyte patch for transplantation, preserving the spheroid-forming activity is crucial to provide a survival advantage



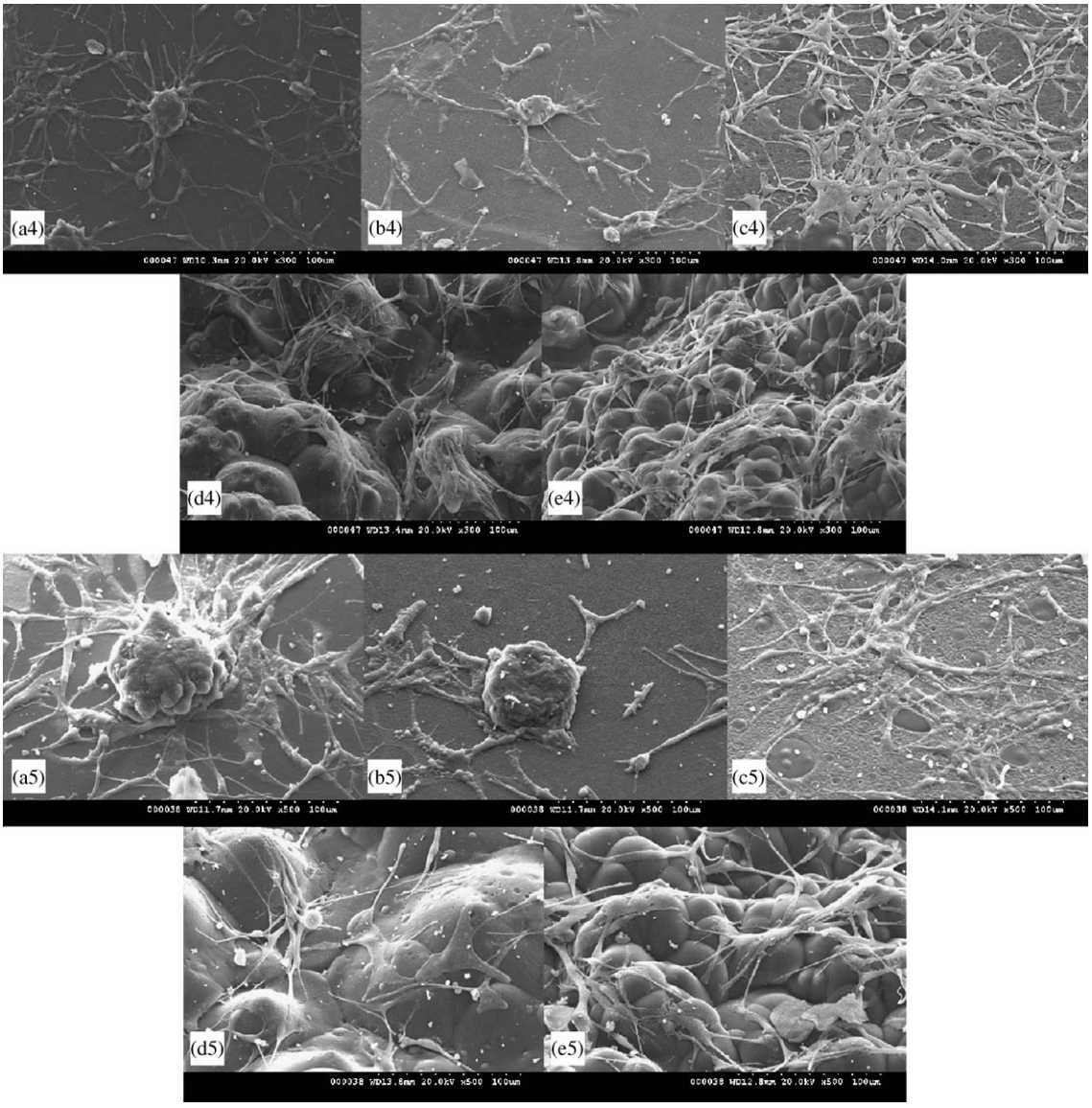


Fig. 4. (*Continued*)

while preparing a blended membrane to enhance the cell growth and mechanical properties [9].

For development of efficient methods to culture cells into spheroids, surface modification of biomaterials is usually required [30]. However, the mechanical properties of biomaterials cannot be significantly modified at the same time. In this work, we investigate the effect of blended membranes on the spheroid formation of cells. To our knowledge, this is the first time that the effect of blended membranes on the spheroid formation is examined experimentally.

Nylon is chosen in this study because human melanocytes fail to form spheroids on it. Judging from the MTT conversion tests and cell morphology at 4 h after seeding (Figs. 5 and 4(e1)), melanocytes show better adhesion to nylon as compared with that to chitosan membrane. During the culture period for 5 days, no spheroid formation is observed on nylon surface. The trend is

Fig. 4. Cell morphologies of melanocytes cultured on prepared membranes from 4 h to 7 days after seeding. (a1, b1, c1, d1, e1) Cell morphologies 4 h after seeding. Melanocytes display a round or bipolar morphology on chitosan and NC75. Cell morphologies become more dendritic as nylon content increases. (Magnification $\times$ 300) **(**a2, b2, c2, d2, e2**)** Cell morphologies after 1 day in culture. A flat dendritic morphology can be observed on all membranes. No spheroid formation is observed. (Magnification $\times$ 300) **(**a3, b3, c3, d3, e3**)** Cell morphologies after 3 days in culture. Melanocyte spheroids can be observed on chitosan and NC75 membranes. (Magnification $\times$ 300) **(**a4, b4, c4, d4, e4**)** Cell morphologies after 5 days in culture. Melanocyte spheroids can only be observed on chitosan and NC75 membranes. (Magnification $\times$ 300) **(**a5, b5, c5, d5, e5**)** Cell morphologies after 7 days in culture. Melanocyte spheroids can only be observed on chitosan and NC75 membranes. (Magnification $\times$ 500) (a1–a5: chitosan; b1–b5: NC75; c1–c5: NC50; d1–d5: NC25; e1–e5: nylon).

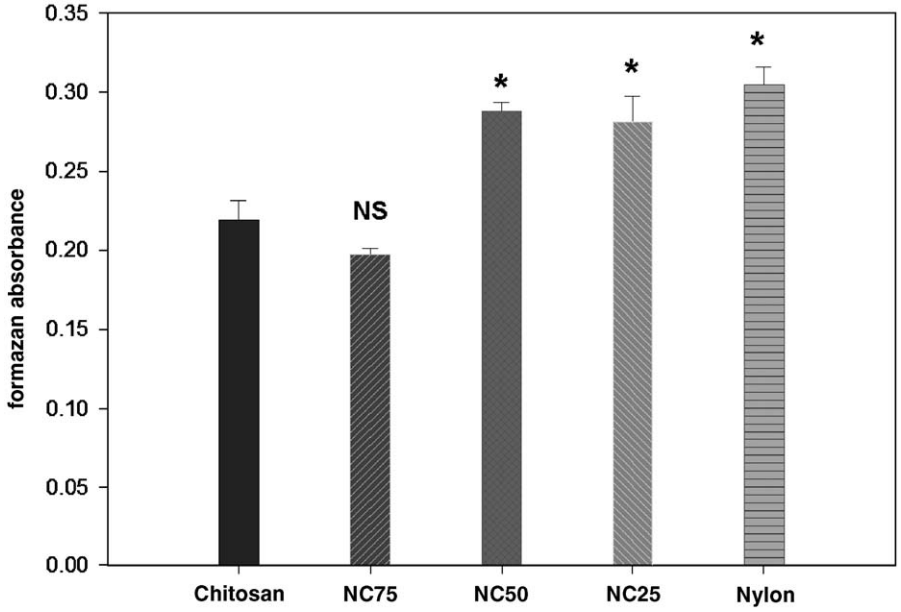


Fig. 5. MTT assays of melanocytes on prepared membranes after 4 h in culture. Significant higher absorbance can be detected in NC50, NC25 and nylon membranes, indicating that cells have better adhesion to these membranes. Errors bars reflect standard deviations calculated from four independent experiments. Student's t test is performed against chitosan group for comparison ($^*p<0.05$; NS: non-significant).

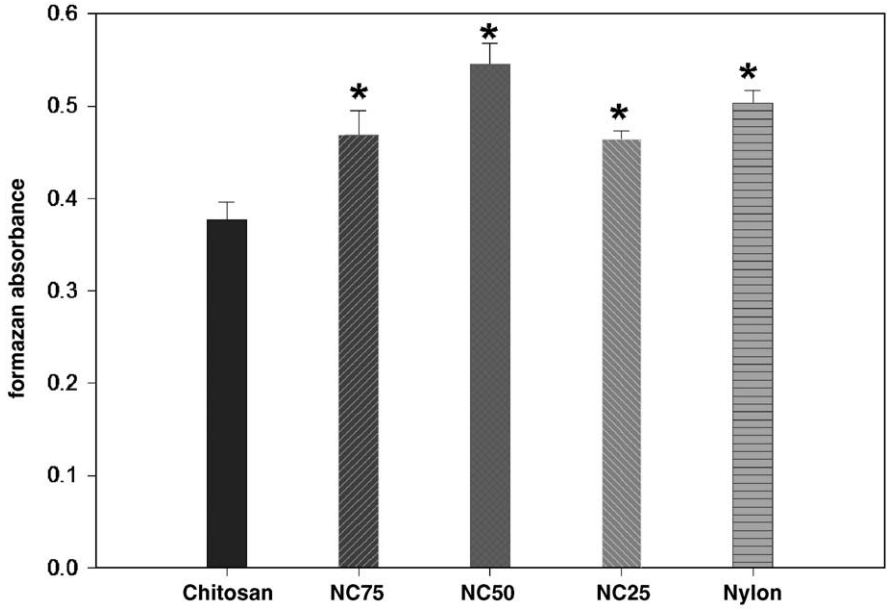


Fig. 6. MTT assays of melanocytes on prepared membranes after 7 days in culture. Significant higher absorbance can be detected in NC75, NC50, NC25 and nylon membranes, indicating that cells have enhanced growth on pure nylon membranes and all blended membranes in comparison with that on chitosan membranes. Errors bars reflect standard deviations calculated from four independent experiments. Student's t test is performed against chitosan group for comparison ($^*p<0.05$).

consistent with our previous report in which melanocytes displayed better adhesion to TCPS and failed to form multicellular spheroids on it [8].

Why melanocytes form spheroids on chitosan is not clarified in our previous work [8]. In order to interpret the behavior of melanocytes on chitosan surface, i.e. spheroid formation, we have proposed the formation of melanocyte spheroids is mediated by a balance between two competing forces: the interactions of cell–substrate and cell–cell [8]. When cell–cell interaction exceeds cell–substrate interaction, cells may aggregate into multicellular spheroids. By blending two materials with different effects on melanocyte spheroid formation, chitosan promotes spheroid formation while nylon not, we can examine the hypothesis.

According to our previous work, nylon and chitosan are microscopically immiscible [15]. Further, differential scanning calorimetry (DSC) and wide-angle X-ray diffraction analysis also showed that blending chitosan with nylon does not obviously affect the crystallization of nylon. Hence, we can expect that nylon and chitosan on the surface of the blended membranes still preserve their original property, i.e. interaction with cells. Judging from the cell morphology and MTT assay at 4 h after seeding, melanocytes have better adhesion to pure nylon membrane than that to pure chitosan membrane. Similar to TCPS [8], the better adhesion of melanocytes to nylon may reflect a stronger cell–substrate interaction, resulting in the inhibition of spheroid formation. When more nylon is incorporated into chitosan, the relative surface area occupied by nylon increases. Since melanocytes have very long dendrites, the cells contact multiple chitosan and nylon phases at a time. The interaction between a single cell and the substrate is the sum of the interaction with the total surface that a cell contacts at a time. Theoretically, we can expect that when nylon increases, the overall interaction of a melanocyte with the substrate increases. Once the increasing cell–substrate interaction overcomes the cell–cell interaction, the formation of melanocyte spheroids will be inhibited.

Examination of the cell morphology and MTT conversion at 4 h after seeding reveals that the cell adhesion is ameliorated as more nylon is incorporated into the blended membrane. Hence, the interaction of cell–substrate becomes stronger. By examining the cell behavior on the prepared membranes during the further culture period, melanocyte spheroid formation can only be observed on pure chitosan membranes and membranes composed of only 25% nylon (NC75). When nylon content increases further, spheroid formation is inhibited. This indicates that the cell–substrate interaction in the case of NC50, NC25 and pure nylon membranes exceeds the cell–cell interaction and thereby preventing cells from aggregating into spheroids. In short, the results substantiate our hypothesis that the spheroid formation of cells on biomaterials is dependent on the balance of cell–cell interaction and cell–substrate interaction.

In addition to the cell adhesion and spheroid formation, other important properties of the blended membranes are investigated in this work as well. Several important properties of the prepared membranes also show a similar proportional change to increased nylon content, including surface undulation and membrane opacity. Morphologically, pure chitosan membrane has a dense plane surface while pure nylon membrane is characterized by a particulate surface. When nylon is blended into chitosan, the surface of the blended membranes becomes progressively undulating. In addition to the chemical and physical properties of the membrane surface, one can argue that the undulating topology can prevent melanocytes from migrating and serve as a key factor in inhibiting spheroid formation. In the case of NC50 membrane, the surface is still generally flat and melanocyte dendrites span multiple nylon and chitosan phases. However, melanocytes fail to aggregate on it. When NC25 and pure nylon membranes are taken into account, we can see that cells are mainly located in the dells of the surface 4 h after seeding. This can possibly be due to the gravity effect during seeding. Upon further culture period for up to 7 days, both the dells and protruded areas of the membrane surfaces are evenly occupied by melanocytes. This suggests that, though the surface is increasingly undulating, melanocytes can still move on it. Therefore, the undulating surface may not be the key factor in preventing cells from aggregating into spheroids. To further clarify this point, we also prepared pure nylon membrane with a flat surface for melanocyte culture. Melanocytes remained monolayered and dendritic on it and no spheroid formation was observed (data not shown).

As for clinical application, the transparency of the prepared membranes is advantageous. The results also show that the opacity of the membrane is proportional to the nylon content. For cell observation during culture, only pure chitosan and NC75 membranes allow direct observation under inverse phase contrast microscope (data not shown). Appropriate transparency cannot be maintained with higher nylon content, such as NC50 and NC25 membranes.

Interestingly, the trend of cell growth is not proportional to the increasing nylon content. Judging from cell growth during the culture period for 7 days, as nylon content increases, cell growth rate first increases in NC75 and NC50 groups and then slightly decreases in NC25 and nylon groups. Thus, the prepared nylon and chitosan-blended membranes exerted different effects on spheroid formation and cell growth. Recently, we proposed that the cell adhesion is regulated by a different mechanism from that of cell activation and cell growth on biomaterials [15,31]. The observation here further solidifies our previous report [15].

5. Conclusion

In this study, we examined the effect of blended membranes composed of immiscible polymers on the spheroid formation, adhesion and growth of human melanocytes. Cell adhesion and spheroid-forming activity are correlated with the relative bulk ratios of polymers in the blended membrane. The opposite trends for cell adhesion and spheroid formation substantiate our hypothesis that spheroid formation on biomaterials depends on the balance of two competing forces: cell–cell interaction and cell–substrate interaction. Multicellular spheroids can form on biomaterial surfaces when cell–cell interaction exceeds cell–substrate interaction. Our work can serve as a model to investigate the relative strengths of cell–cell and cell–substrate interactions and also pave way to design blended membranes with desired physical properties while preserving the spheroid-forming activity of cells in the field of tissue engineering.

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