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In Vitro Cytotoxicity Characterisation of Keratin Protein for Tissue Engineering Applications

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Abstract: In order to evaluate the application of keratin protein in its natural form for tissue engineering, wool fibres were subjected to *in vitro* cytotoxicity tests. Non-woven felts of wool fibre were plasma treated to increase its wettability. These felts were also tested for cytotoxicity. Extraction test as laid down in European and international standards (ISO/EN 109935 Guidelines) was performed on both the wool fibre samples as well as the plasma treated wool felt samples. L 929 cells which are mouse kidney fibroblast cells which are low maintenance, rapidly growing adherent cells forming confluent carpet formation were used. The test sample is non-cytotoxic if the percentage viability of 100% test sample is >70%. Cell viability percentage of 87.35 and 89.38% for wool fibres and the plasma treated felt, respectively showed no cytotoxic character. The results suggest that wool fibres can be used as a biomaterial for tissue engineering applications.

Key words: Cytotoxicity, Keratin, L 929 cells, wool fibre, keratin, plasma treated

INTRODUCTION

As the biological structural component of skin (soft keratins) and of nails, claws, hair, horn, feathers and scales (hard keratins) pure keratin comprises up to 90% by weight of wool (Cardamone *et al*., 2009). Keratin is extracted from various sources including wool.

Keratin scaffolds have been fabricated in the form of films, sponges and fibres. Keratin biomaterials possess many distinct advantages over conventional biomolecules including a unique chemistry afforded by their high sulphur content, remarkable biocompatibility, propensity for self-assembly and intrinsic cellular recognition (Rouse and Van-Dyke, 2010). Scaffold fabricated from keratin and agar showed good biocompatibility against the myofibroblast cell line (C2C12) and have promising characteristics for tissue engineering applications. Good degradability and biocompatibility properties, also favour its competent candidature to construct a porous scaffold for biomaterial application (Kumar and Gupta, 2015).

The hydrophilicity of a material is an important factor for cell adhesion and growth and improved surface hydrophilicity of materials will improve the interactions between the materials and cells for eliciting controlled cellular adhesion and maintaining differentiated phenotypic expression (Arima and Hiroo, 2007; Lei *et al*., 2015). The higher permeability significantly improved the wetting properties of the hydrophobic scaffolds and increased the settling speed of cells upon static seeding of immortalised mesenchymal stem cells (Melchels *et al*., 2010).

Cell attachment, cell substrate interactions are likely to be a function of the substrate chemistry (Noth *et al*., 2005). Optimum cell attachment is observed on surfaces with low to moderate levels of hydrophilicity (Lydon *et al*., 1985). Hydrophobic surfaces strongly denature adsorbed proteins (Sigal *et al*., 1998).

To enhance corneal epithelial cell attachment and growth an ammonia plasma treatment has been applied to artificial corneas fabricated from poly (hdroxyethyl methacrylate) (Sipehia, 1993). In another study microwave-induced argon plasma was used to modify and sputter-coat the surface of nanofibrous silk fibroin scaffolds with gold/platinum to enhance the attachment and proliferation of the human articular chondrocyte cultures (Baek *et al*., 2008). The increase in cell growth on a technical textile (ECTFE) can be linked to an increase in wettability and the introduction of oxygen containing functional groups (Vohrer, 2007). Plasma can be used to improve the wettability of fabrics. The magnitude of wettability is higher for the GD treated sample when compared to the corresponding data determined for BD treated wool (Thomas, 2007).

The suitability of any biomaterial for any application in tissue engineering has to be studied thoroughly from the point of view of all the requirements of the scaffolds,

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i.e., toxicity, biodegradability, porosity, difficulties in fabrication, tensile strength and the ease of its preparation into 3D constructs (Baliga and Borkar, 2016a, b). The cytotoxic nature of any material is tested by the guidelines laid down in European and International standards (ISO/EN 109935) (Baliga and Borkar, 2016a, b).

Although, a lot of research is found on the application of extracted keratin protein and the importance of hydrophilicity of the biomaterial for tissue engineering applications, researchers did not examine the biocompatibility of wool fibres and therefore the examination of the cytotoxic character of wool fibres and plasma treated wool fibre structures is important.

MATERIALS AND METHODS

Fine merino wool (16-17 μm) fibres were purchased from Little Barn, USA.

Preparation of the wool felt: The scaffolds were prepared by the wet felting method. About 5 g of the wool fibre was weighed and hand laid layer by layer in a mould of size 12×12×0.1 cm, approximately 0.34 g/cc. The fibre tuft was gently pulled from the top and was placed uniformly in the mould. Each layer was placed at 90° to the previous layer and a batt was prepared. This batt was then placed on a bubble wrap and covered with a piece of netting. Soapy water at 80-90°C was sprinkled through the net without over wetting the fibres. The wool is pushed down by hand and the water is allowed to be absorbed by the fibres. Without disturbing the fibres the top of the net was gently rubbed using a circular motion for a few minutes. Using a roller the entire assembly was rolled. It was rolled backward and forward for a minute. The felt is carefully unrolled and the process is repeated by turning the felt through 90°. Repeat rolling till the wool is felted. It can be ensured by the pinch test. Pinch the felt between the thumb and the forefingers. If the layers fall apart, the felt is not ready. The rolling process is repeated till the felt is ready. The felt was rinsed with cold water and dried.

Plasma treatment of the wool felt: The plasma system works on the principle of dielectric barrier discharge generated between the top and the bottom electrodes. The system works with the dielectric barrier discharge and it operates in continuous mode where online treatment of fabric in plasma zone is possible. It consists of set of four planar electrodes aligned horizontally in series. Another set of four planar electrodes are placed at the bottom of above electrodes. The length of plasma zone (total length of electrodes in the direction of fabric movement) is 12 cm and width is 55 cm. The spacing between top and bottom electrodes can be manually adjusted depending on the thickness of the fabric. The flow rate for Helium and Oxygen was kept at 5 L/min and 1 L/min, respectively. Helium and Oxygen were mixed together and the mixture was used as a carrier gas for plasma generation. The flow rates of the gases were controlled by manual flowmeters.

This mixture was supplied to the electrode system where the electric power was applied to generate the non-thermal plasma at atmospheric pressure. The discharge power was 3.5 kW. The dwell time of the sample in the plasma zone was 60 sec and the inter-electrode spacing was maintained at 4.5 mm during the treatment.

Contact angle measurement: The wettability of the scaffold was measured at BTRA, Mumbai by the contact angle measurement, using sessile drop method on "Easy Drop" standard drop analysis system of KRUSS GmbH, Germany, equipped with high speed camera IEEE1394b interface.

***In vitro* cytotoxicity test:** International Standard ISO 10993-5:2009(E), biological evaluation of medical devices, part 5: tests for *in vitro* cytotoxicity are designed to determine the biological response of mammalian cells *in vitro* using appropriate biological parameters. An extract of the scaffold sample is tested for cytotoxicity. It measures cell viability via. metabolic activity. At the end of the exposure time, the evaluation of the presence and the extent of cytotoxic effect are assessed. It signifies Biological compatibility of the test material and its potential to cause cell damage. Yellow water-soluble MTT 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) is metabolically reduced in viable cells to a blue-violet insoluble formazan. The number of viable cells correlates to the colour intensity determined by photometric measurements after dissolving the formazan in alcohol. The method makes use of L 929 cells. Cells are seeded into 96-well plates and maintained in culture for 24 h. They are then exposed to the test compound over a range of concentrations. After 24 h exposure, the formazan formation is determined for each treatment concentration and compared to that determined in control cultures. For each treatment the percentage inhibition of growth is calculated.

About 1 g of representative sample was sterilized at 121°C for 15 min to which 10 mL complete MEM medium was added (0.01 g/mL) and incubated at 37°C in 5% carbon dioxide for 24 h.

L929 cells seeded in 96 well plates at a concentration of 10,000 cells per 100 μL of MEM culture medium per well were maintained in culture for 24 h to form a semi confluent layer and were exposed to the test material over a range of 10-100% extract in triplicates. After 24 h growth microscopic observations reveal the viability of cells in varying percentage extract. This can be translated into colour complex by MTT assay. Liquid medium was eluted from 96 well plate and MTT reagent added incubated at 37°C in 5% carbon dioxide for 2 h, followed by DMSO and colour development. Cell viability correlated with intensity of colour measured by photometric measurements by OD measurements at 570 nm.

A decrease in number of living cells results in a decrease in the metabolic activity in the sample. This decrease directly correlates to the amount of blue-violet formazan formed as monitored by the optical density at 570 nm. To calculate the reduction of viability compared to the blank the following equation used is:

$$\text{Viability (\%)} = \frac{OD_{570e}}{OD_{570b}} \times 100$$

Where:

OD_{570e} = The mean value of the measured optical density of the 100% extracts of the test sample

OD_{570b} = The mean value of the measured optical density of the blanks

RESULTS AND DISCUSSION

Hydrophilicity of the sample after plasma treatment: The surface hydrophobicity can be assessed by measuring contact angle through water spread of a droplet on a surface. The lower the contact angle, the more hydrophilic the surface is.

An average contact angle reading of 139.5° was recorded for the untreated wool felt sample. The high reading indicates that the wool fibres are hydrophobic in nature (Fig. 1).

Plasma treatment of wool ruptures the epicuticle of the wool fibres and it was expected that the wettability of the scaffold would increase. Wettability of the scaffold could influence the cell adhesion and proliferation.

For the plasma treated sample the contact angle could not be measured. The time to absorb the drop in case of for the plasma treated sample it was 30-40 sec.

It indicates that the plasma treatment improves the hydrophilicity of the sample drastically. As suggested by previous research, the cell interactions are a function of the wettability of the scaffold.

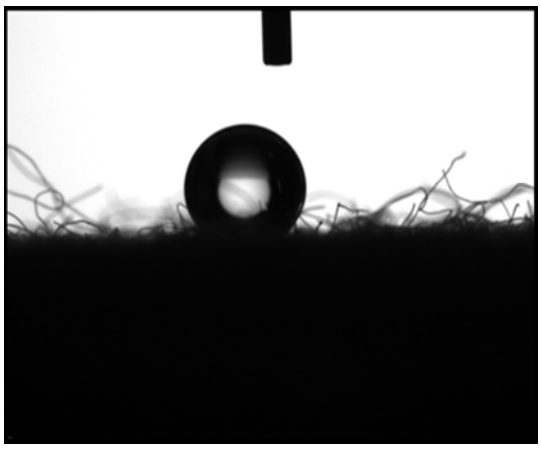

Fig. 1: Contact angle measurement for the untreated wool fibre felt

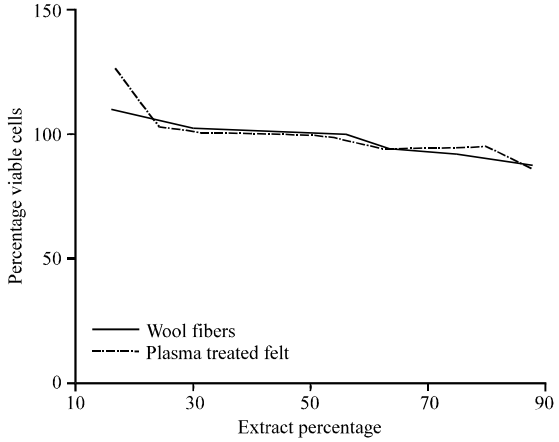

Fig. 2: Graph showing the cytotoxicity results of untreated wool fibres and the plasma treated felt samples (*In vitro* cytotoxicity)

***In vitro* cytotoxicity test:** The *in vitro* cytotoxicity test was carried out for both wool fibres and the plasma treated wool felt (Fig. 2).

The lower the cell viability (%) value, the higher the cytotoxic potential of the test item is. If viability is reduced to <70% of the blank it has a cytotoxic potential. The 50% extract of the test sample should have at least the same or a higher viability than the 100% extract, otherwise, the test should be repeated. If the percentage viability of 100% test sample is ≥70% it is non cytotoxic.

It is observed that the cell viability% values for both wool fibres and the plasma treated felt are 87.35 and 89.38%, respectively. Both the values are higher than 70% (Table 1).

Table 1: Cytotoxicity results of untreated wool fibres and the plasma treated wool felt

Samples	10%	20%	30%	40%	50%	60%	70%	80%	90%	100%
Wool fibres	126.25	103.43	100.64	100.64	100.96	98.07	95.60	95.17	95.17	87.35
Plasma treated felt	111.00	105.46	103.96	102.60	102.46	101.71	94.85	93.14	90.56	89.38

CONCLUSION

The untreated wool fibre felt was hydrophobic. The wettability of the wool fibre felt after plasma treatment has increased drastically.

The wool fibres are non-toxic under the extract testing for the cells of cellular culture. The plasma treated felt is also found to be non-cytotoxic.

Wool comprises of 90% of keratin protein which has been widely researched. Wool fibre has a huge potential in the area of tissue engineering.

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