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# A chemically defined surface for the co-culture of melanocytes and keratinocytes

Paula Clare Eves[a], Alison J. Beck[a], Alex G. Shard[a], Sheila Mac Neil[a,b,*]

[a]*Department of Engineering Materials, University of Sheffield, Sir Robert Hadfield Building, Mappin Street, Sheffield, S1 3JD, UK*
[b]*Section of Human Metabolism, Division of Clinical Sciences (North), Northern General Hospital, Herries Road, Sheffield, S5 7AU, UK*

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**Abstract**

Patients with stable vitiligo can be helped surgically using transplantation of autologous cultured melanocytes, but there is a need for a culture methodology that is free from xenobiotic agents and for a simple way of delivering cultured melanocytes to the patient to achieve pigmentation with good wound healing. The aim of this study was to develop a chemically defined surface, suitable for the co-culture of melanocytes and keratinocytes which could be used in the future for the treatment vitiligo patients to achieve both restoration of pigmentation and good wound healing. Two keratinocyte growth media and two melanocyte growth media were compared; two of these were serum free. Cells were seeded on a range of chemically defined substrates (produced by plasma polymerisation of acrylic acid, allylamine or a mixture of these monomers) either as mono- or co-cultures. Melanocytes and keratinocytes attached and proliferated on both acid and amine substrates (without significant preferences), and co-cultures of cells proliferated more successfully than individual cultures. One media, M2, which is serum free, supported expansion of melanocytes and to a lesser extent keratinocytes on several plasma polymer substrates. In conclusion, these data indicate that a combination of a chemically defined substrate with M2 media allows serum-free co-culture of melanocytes and keratinocytes.


*Keywords:* Vitiligo; Plasma polymerisation; Acrylic acid; Allylamine; Keratinocytes; Melanocytes; Skin tissue engineering

## 1. Introduction

Vitiligo describes depigmentary skin disorders of uncertain pathogenesis that affect 0.5–2% of most populations. It is thought that the end result of loss of melanocytes may represent several different vitiligo conditions each with a different aetiology [1–4]. While not life threatening, in dark-skinned populations the presence of unsightly chalk-white lesions of variable sizes and locations on the skin, can lead to serious psychological problems in daily life [5]. In addition, the white areas which lack melanin pigmentation are very sensitive to sun exposure and can easily burn.

Although very common, vitiligo remains a therapeutically challenging and difficult disorder to treat. Different therapeutic modalities have been used to achieve pigmentation from UV (e.g. PUVA, narrow-band UVB), laser and immunomodulatory therapies (reviewed in [6]), to surgical procedures such as suction epidermal grafting, punch minigrafting, cultured melanocyte suspensions and cultured epithelial autografts (CEAs) [7–10]. In our laboratory over the past decade, CEAs (containing "passenger" melanocytes) have been used to successfully treat badly burned patients. However, when we adapted this method to treat vitiligo patients, we had very limited success with only one patient out of five achieving pigmentation, although all had good wound healing [11]. Upon investigation of the fate of the "passenger" melanocytes within these epithelial autografts, Phillips et al. demonstrated that as keratinocytes achieve high density, as occurs in these

*Corresponding author. Department of Engineering Materials, University of Sheffield, Sir Robert Hadfield Building, Mappin Street, Sheffield, S1 3JD, UK. Tel.: +44 114 222 5995; fax: +44 114 222 5943.
*E-mail address:* s.macneil@sheffield.ac.uk (S. Mac Neil).

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CEAs prior to grafting, keratinocytes downregulate both melanocyte number and melanocyte pigmentary function [11].

However, the strategy of grafting keratinocytes and melanocytes as a co-culture remains an attractive alternative to transplanting melanocytes alone. The addition of keratinocytes ensures rapid healing which reduces the risk of scarring following dermabrasion, whilst the melanocytes pigment the transplanted areas. Furthermore, co-cultures of keratinocytes with melanocytes have, until recently offered a viable way of isolating and growing melanocytes in vitro for clinical use, without the need for phorbol esters, pituitary extract and serum, all of which are routinely used in the in vitro culture of melanocytes. It is important to avoid these factors as phorbol esters are co-carcinogens for the skin, and bovine sera and pituitary extract may contain foreign proteins and slow viruses which can cause allergic reactions and have been linked to diseases such as bovine spongiform encephalopathy (BSE) [12]. In response to the need to avoid transmission of xenobiotic materials and potential carcinogens, studies by Olsson and colleagues have shown that melanocytes can be isolated in vitro in a medium free from sera, phorbol esters and pituitary extract. Once transplanted, repigmentation of vitiligo sites can be successfully achieved using these melanocytes [12–15]. The method of delivery in all these studies involved a suspension of melanocytes being added directly to the recipient site, which was then covered with a Millipore-net or collagen dressing followed by a media moistened gauze and a Tegaderm dressing. When this technique was used by Van Geel and colleagues [16], however, they reported difficulty in keeping the cell suspension on curved areas at the desired recipient site, due to its fluidity.

A key interest within our group over the last few years has been the development of a chemically defined surface which not only supports the growth of keratinocytes, but can be used clinically to deliver adherent autologous keratinocytes for the treatment of burns and chronic ulcers [17–21]. This work demonstrates that chemically engineered surfaces can be used successfully to transfer cells in a clinical environment. More recently we have shown that serum-free culture can be achieved for keratinocytes if fibroblasts are used instead of serum to support keratinocyte expansion [22,23]. We have also shown that a chemically defined surface can alter the growth profile of melanocytes cultured in different media. For example, when melanocytes (cultured as ''passenger'' melanocytes within a keratinocyte culture) were grown on a collagen I surface, melanocyte survival was greatest with Green's medium (containing 10% serum) rather than a defined keratinocyte medium (KDM). Whereas, when cells were cultured on a chemically defined substrate (a nitrogen-containing plasma polymer (pp) surface), melanocyte number was greatest in KDM rather than Green's medium [24]. This latter study demonstrates that the substrate itself (as well as the culture medium) can play a major role in influencing the proliferation of cells and that the use of chemically defined surfaces may contribute to eliminating the need for animal-derived products and serum, hence reducing the risk of disease transmission for patients.

With this in mind the aim of the current study was to further develop a chemically defined surface (using plasma polymerisation) for the co-culture of keratinocytes and melanocytes for future use in the grafting of vitiligo patients.

We report that keratinocytes and melanocytes can be co-cultured on a range of chemically defined substrates (e.g. acrylic acid (AC), allylamine (AA)) and that cell preference for an engineered surface can be influenced by both media and cell/cell interactions. From this work we have identified substrates which can be used with commercially available serum-free media for keratinocyte and melanocyte co-culture. We hope this will now enable us to develop a new approach for treating patients with stable vitiligo.

## 2. Methods

### 2.1. Materials

Dulbecco's modified Eagle's medium (DMEM) with phenol red pH indicator, glucose, glutamax and pyruvate (1 ×), Ham's F12 media, keratinocyte defined media (KDM), glutamine, penicillin, streptomycin, fungizone, Triton ×100, bovine pituitary extract (BPE) and trypsin/EDTA were all obtained from Gibco BRL, Paisley, UK. Melanocyte growth medium M2 was purchased from Promocell GmbH, Heidelberg, Germany. Defined trypsin inhibitor (DTI) was obtained from Cascade Biologics, Mansfield, UK. Trypsin (0.1% w/v) was purchased from Difco Laboratories, Detroit, USA. Fetal calf serum (FCS) was from GlobePharm Limited, Esher, UK. Phosphate-buffered saline (PBS) tablets were purchased from Oxoid Ltd. Basingstoke, UK. Tissue culture plastics were obtained from Corning Costar Corporation, Cambridge, USA. Tri-iodothyramine, epidermal growth factor (EGF), hydrocortisone, adenine, insulin, transferrin, cholera toxin, phorbol 12-myristate 13-acetate (PMA), geneticin, MCDB 153 basal medium, nystatin, MTT, ethylene glycol monoethyl ether (Cellusolve), and trypan blue were from Sigma Chemicals Ltd. Poole, UK. Tween and paraformaldehyde, were purchased from BDH, Merk Ltd. Poole, UK. Biotinylated anti-goat IgG and Streptavidin-FITC were from Vector Laboratories, Burlingame, USA. Ten percent phosphate-buffered formaldehyde was obtained from Genta Medical, York, UK. DakoCytomation LSAB 2 system-AP, Dako fuchsin substrate–chromogen system, Dako protein block—serum free, glycergel and rabbit-derived S100 antibody were purchased from Dako Corporation, Carpintera, USA. AA and AC were supplied by Aldrich