



# Cell sheets prepared via gel–sol transition of calcium RGD–alginate



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## ABSTRACT

The formation of layered tissues through the use of cell sheet harvesting has recently emerged as a potentially viable approach for clinical tissue engineering applications. Since the demonstration of effective cell sheet formation using temperature responsive substrates, a number of different stimuli have been utilized to facilitate cell sheet detachment. Each approach has differing advantages and disadvantages. Herein we demonstrate the ability of calcium alginate hydrogels to function as an effective substrate for cell sheet formation. By conjugating the integrin binding peptide sequence RGD to the alginate and crosslinking it with calcium ions, the hydrogel formed supported the attachment and growth of both 3T3 fibroblasts and human corneal epithelial cells (HCECs). Once the cells had grown to confluence, exposing the calcium alginate to the chelating agent citrate caused the release of a consolidated cell sheet. When HCEC sheets were stacked, the cell layers adhered to each other and the cells began to integrate.

### Statement of significance

Herein we describe a simple and inexpensive process for creating cell sheets using the ability of calcium alginate hydrogels to be dissolved under mild conditions. The alginate was first modified to possess cell attachment sites and in this demonstration of feasibility we employed an RGD peptide, but other peptides could be readily attached. The modified alginate was then formed into stable hydrogels through a drying and re-hydration step, and used as a substrate to grow confluent cell sheets of human corneal epithelial cells and 3T3 fibroblasts as examples. The cell sheets were released through chelating the calcium using citrate. The cell–cell connections were retained following this release and the cell sheets interconnect and grew following being stacked.

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## 1. Introduction

In tissue engineering, the general practice is to create a three-dimensional porous scaffold using a biodegradable polymer that supports cell attachment and growth and then seed this scaffold with cells. This strategy has a number of potential disadvantages. The volume initially occupied by the polymer is eventually opened by its degradation; in the meantime, however, the cells have generated a large amount of extracellular matrix (ECM) that has accumulated on the surface of the polymer. The ECM deposited impedes the migration of the cells into the void spaces created by the polymer degradation. Thus, this approach is limited to tissues with low cellularity, such as bone and cartilage [1]. An approach that does not rely on a polymer scaffold may be required

to produce cell dense tissues such as the corneal epithelium and the skin. Such an approach is the formation of cell sheets, which retain their extracellular matrix and which can be combined to form tissue-like structures.

The first approach to address this problem effectively was a temperature-responsive cell culture process that utilized poly(*N*-isopropylacrylamide) (PNIPAm) grafted to the surface of a tissue culture plastic plate [2]. This polymer exhibits a hydrophobic surface at temperatures greater than 32 °C, which effectively allows for protein adsorption from culture media. Cells attach to these adsorbed proteins and then proliferate and secrete ECM that accumulates between them and the underlying PNIPAm. Once the cells have reached confluence, reducing the temperature to below 20 °C causes the PNIPAm to undergo a transition and become hydrophilic. This change in water affinity of the polymer causes detachment of the cells and their accumulated ECM in a consolidated, contiguous sheet. Once the cell sheet has been detached, the temperature can be raised again and the process repeated to form another cell

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sheet. This approach has been improved upon since its inception, and tissues made from this technology are currently in clinical trials for the treatment of myocardial infarct as well as for corneal implants [3].

Others have formed cell sheets via an electrochemical reduction methodology [4–6]. Inaba et al. prepared an alkanethiol containing a terminal adhesive peptide such as RGD, and attached them to a gold surface forming a monolayer [5]. The cells attach to this monolayer and grow to confluence and accumulate ECM between them and the underlying monolayer. Application of an electric field for 10–20 min caused detachment of the monolayer from the underlying gold surface releasing the cell sheet. Yeo and Mrksich prepared alkanethiolate monolayers on gold substrates, and the alkanethiolates were terminated with cell peptide ligands coupled through redox reactive chemical groups [4]. Application of a voltage in this case caused a cleavage of the chemical coupling attaching the cell sheet to the alkanethiolate monolayer, releasing the cell sheet. Guillaume-Gentil et al. coated titanium dioxide surfaces with a polyelectrolyte multi-layer film composed of serial layers of poly(L-lysine) (positively charged layer) and hyaluronic acid (negatively charged layer) followed by a surface layer of RGD-grafted poly(L-lysine)-graft-poly(ethylene glycol) (PLL-PEG) (positively charged layer). Cells were seeded onto these surfaces and grown until confluent, following which cell sheet detachment was achieved by applying 1.3–1.5 V for 30 min. The applied voltage generates protons at the surface, lowering the microenvironmental pH and thereby disrupting the electrostatic interactions bonding the polymer layers together.

In a different polyelectrolyte multi-layer (PEM) approach, Zahn et al. prepared 2 PEM-blocks [7]. The first block consisted of a crosslinked poly(ethylene imine-(hyaluronic acid/poly(L-lysine)))<sub>10</sub> multilayer (i.e. a first layer of poly(ethylene imine) followed by ten alternating layers of hyaluronic acid (HA) and poly(L-lysine) (PLL)). Following formation of the ten layers, the block was cross-linked using EDC/NHS. On top of this block were adsorbed a few bilayers of HA/PLL, and then the surface was coated with a layer of fibronectin, which is negatively charged at pH 7.4. The cells were cultured on this surface, and then released following incubation in 5 mM ferrocyanide for 5 min. Ferrocyanide is used as a food additive and thus considered nontoxic, and erodes the HA/PLL multilayers due to competitive ionic displacement.

Light has also been used as a means of inducing release of an established cell sheet. For example, 365 nm UV light can be employed to induce a change in titanium surface hydrophobicity [8] or cleave an integrin binding peptide sequence such as RGD from a backbone polymer [9]. Strategies based on the use of UV light run the risk of inducing cell damage, as has been noted for photo-encapsulation of cells [10]. For this reason, Edahiro et al. grafted spiropyran onto poly(*N*-isopropylacrylamide) that was incorporated into a cell culture surface [11]. Irradiation of spiropyran by UV light enhanced cell attachment to the poly(*N*-isopropylacrylamide), providing a surface on which they could proliferate and form a sheet. To release the cell sheet, the surface was irradiated with visible light (400–440 nm, 24 mW/cm<sup>2</sup>) for 5 min, followed by incubation at 37 °C for 2 h, followed by cooling on ice for 20 min and washing with ice cold phosphate buffered saline.

Finally, other research groups have used substrates for cell attachment that are degraded by non-proteolytic enzymes. These approaches include the formation of covalently linked surface layer of cellulose with conjugated fibronectin [12] or covalently formed gels of polysaccharides such as carboxymethylcellulose–tyrosine [13] and alginate–tyrosine [14] cross-linked in the presence of a cell adhesive substrate such as gelatin. Cells are then seeded on top of the immobilized or gelled polysaccharide gel surface and grown to confluence. A contiguous cell sheet is obtained by incubating the surface with an enzyme specific for the polysaccharide

used (e.g. cellulase for cellulose or alginate lyase for alginate gels) for a period of from 25 to 30 min at 37 °C.

Although each of these approaches has demonstrated the ability to form a contiguous cell sheet, they also have potential limitations. In the PNIPAm approach, it is critical to control the grafting density and molecular weight of the grafted PNIPAm macromolecules in order to obtain cell adhesion at 37 °C as well as cell sheet detachment. If the grafted layer possesses a thickness greater than 30 nm, the cells will not adhere, while if the layer thickness is less than 15 nm, the cells will not detach from the surface [1]. The polymer is deposited onto the surface using e-beam irradiation treatment, which is an expensive process. Further, the detachment process requires from 30 to 70 min and causes changes to cell metabolic processes, while optimal temperature conditions vary from cell to cell; for example, 20 °C works well for endothelial cells but hepatocytes require 10 °C. With the electrochemically induced release approach of Inaba et al. and Mrksich et al., an expensive substrate is required, while the lowered pH generated by the formation of protons and the applied voltage required in the strategy of Guillaume-Gentil et al. are potentially damaging to cells, and the PLL used in the generation of PEMs is considered to be cytotoxic [15]. The photo-responsive systems involve either long-wave UV, which may cause cell damage, or a lengthy process using visible light. Finally, non-proteolytic enzymes must be completely removed to avoid immunological issues upon tissue implantation *in vivo*.

It was the objective of this work to develop a simple and inexpensive process for the generation of a contiguous, viable cell sheet. The process was based on the established ability to liquefy calcium alginate hydrogels using chelating agents such as sodium citrate, which can be achieved without adverse effects on cell viability [16]. Sodium alginate was first reacted with a peptide containing the RGD amino acid sequence to promote cell adherence, then formed into a calcium alginate hydrogel on a Transwell membrane and used as a cell culture substrate. Upon cell growth to confluence, the cell sheet was liberated from the calcium alginate substrate following incubation with a sodium citrate solution (Fig. 1). Herein we demonstrate the feasibility of this approach for the generation of viable, contiguous cell sheets using 3T3 cells and human corneal epithelial cells (HCECs).

## 2. Materials and methods

### 2.1. Materials

Sodium alginate (Protanal LF 10/60) was from FMC Biopolymer, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC-HCl) was from Acros, Canada. N-hydroxysulfosuccinimide sodium salt (purity >98%) (sulfo-NHS) was obtained from Boao-pharma, Inc., USA. 2-(*N*-morpholino)ethanesulfonic acid (MES) and phosphate buffered saline (PBS; without Ca<sup>2+</sup>, Mg<sup>2+</sup>) were obtained from Fisher, Canada. The GGGGRGDS peptide was purchased from CanPeptide, Canada. The hybrid adenovirus 12-SV40 immortalized human epithelial corneal cell line (HCEC) was a gift of Dr. Heather Sheardown of McMaster University, Canada. 3T3-Swiss albino (ATCC CCL 92) cells were purchased from ATCC, USA. Keratinocyte serum-free medium (KSFM, Gibco #17005-042), bovine pituitary extract and epidermal growth factor (Gibco #15140-122), and gentamycin (Gibco #15710-064) were used for HCEC culturing and Dulbecco's Modified Eagle's Medium (DMEM, Gibco #15140-122), fetal bovine serum (FBS, Cat. #SH30396, Fisher Scientific), 100 U/mL penicillin and 0.1 mg/mL streptomycin (1% pen-strep) (Gibco Cat. #15140-122, Life Technologies, Canada) were used for 3T3 cells. CellTracker™ Green CMFDA fluorescent dye (C2925, Invitrogen) and Q-Tracker Orang/Red Cell Labeling Kit (A10198, Invitrogen) for cell staining were obtained from Life

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