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| 13. ABSTRACT (Maximum 200 Words) This program was concerned with developing methods that can pattern the attachment of multiple cell types to a common substrate with absolute control over the position, size, shape and identity of each adherent cell. These methods were developed for use in patterning carcinoma cells and stromal fibroblasts in distinct, non-overlapping patterns for mechanistic studies of the inducible expression of stromelysin. Work in the first two years of this program developed the basis for an electroactive surface that could turn on the immobilization of ligands and hence the attachment of cells. Progress in the third period applied the dynamic substrate to pattern the attachment of two different cell types into a coculture array. This important milestone establishes a method to pattern two (or more) cell types to a substrate with arbitrary control over the geometric patterns of each cell type. Work in the fourth, and final, period has developed an electroactive substrate that can electrically release immobilized ligands. These active substrates will permit control over the time in which one patterned cell type is exposed to a second patterned cell type, and hence provide an important methodology for investigating heterotypic cell-cell interactions in cellular culture systems. |
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Introduction

This Annual Report summarizes the results from our efforts in the fourth, and final, year of the program. The report starts with a description of the background to the overall effort and then provides a technical description of the key results in the final year of activity.

Background. Metastasis of tumors involves a series of regulated adhesions of neoplastic cells with neighboring cells and the extracellular matrix.^{1,2} In order to release their adhesion to the matrix, neoplastic cells express a class of matrix metalloproteinases (MMPs) that degrade the underlying matrix, and allow the intravasation of the tumor cells.^{3,4} This key regulatory event in metastasis has prompted much effort towards the design and screening of inhibitors of the metalloproteinases to afford effective anti-cancer drugs. While the requirement for metalloproteinases in metastasis appears to be general, neoplastic cells have adopted other strategies to enhance the activity of these proteinases.

Chambon and coworkers identified a new metalloproteinase gene expressed in stromal cells of breast carcinomas.⁵ The protein, stromelysin-3 (ST3), is found in carcinomas of the breast, head, and neck, and likely in many other human carcinomas as well.⁵⁻⁸ These workers found that ST3 was expressed in stromal fibroblastic cells immediately surrounding the neoplastic cells of the invasive, but not in the malignant cells. Much evidence now suggests that the carcinoma cells release diffusible factors that in turn induce the expression and release of ST3 by neighboring stromal fibroblasts.⁹ Pei and coworkers reported that ST3, unlike other MMPs, acts as an endopeptidase that cleaves the serine protease inhibitor α 1-anti-trypsin while leaving other matrix proteins unaffected.¹⁰ Proteolysis of α 1-anti-trypsin presumably increases the activity of several proteases that are involved in dissolution of the basement membrane during carcinoma progression.⁸ This mechanism is noteworthy in that the tumor cell does not release the key MMP, but induces neighboring stromal cells to release the protease. The induction of fibroblasts to release ST3 makes possible strategies to combat breast cancer by targeting normal, healthy cells rather than the malignant cells. These strategies may be especially useful for those unstable neoplastic cells that rapidly gain resistance to treatment.

Experimental Studies of Cell-Cell Signaling. Despite much information now available relating ST3 production by stromal fibroblasts to the metastatic potential of neoplastic cells, the interactions between these cells and the tumor cells are not yet well understood. Current research has used single-cell culture, cell transfection techniques, and the study of actual biopsy tissue, but it has not been possible to study the interactions between stromal fibroblasts and tumor cells in a well-defined model system. These studies of the cell-cell communication require a methodology that can pattern the attachment of both cells to a single substrate, with absolute control over the position, size, and identity of each cell. Several groups have demonstrated the use of *patterned* substrates for controlling the positions and shapes of attached cells, but none of these methods can pattern the attachment of multiple cell types with cell specificity—that is, with control over the identity of cells at each adhesive region.¹¹⁻¹⁴

Our program had the primary goal of developing methods to prepare substrates for cell culture wherein the positions for the attachment of different cell types are defined on the substrate. We had proposed to apply these new methodologies for fundamental studies of the carcinoma-induced expression of ST3 by stromal fibroblasts. Our work did not reach this final stage but has proven very significant in developing dynamic substrates that could regulate the presentation of ligands. We have established the use of these substrates for patterning cellular cocultures and have developed a related substrate for releasing attached cells.

We use self-assembled monolayers of alkanethiolates on gold as model substrates for the attachment of cells. These monolayers are the best-available organic surfaces that permit control over interfacial properties.¹⁵ SAMs form spontaneously upon the adsorption of alkanethiols from solution (or vapor) onto a clean surface of gold; the structure of these monolayers is now well-established.¹⁶ The sulfur atoms coordinate to the lattice of the gold surface; the trans-extended alkyl chains are close-packed and tilt approximately 30° from the normal to the surface (Figure 1). The properties of the SAM are determined by the terminal functional group X of the precursor alkanethiol; even complex and delicate groups can be introduced through straightforward synthetic procedures. The properties of the SAM can be further controlled by forming so-called "mixed" SAMs that contain two or more alkanethiolates.^{17,18} SAMs are stable in air for periods of months, though they desorb at temperatures greater than 70 °C, or when irradiated with ultraviolet light in the presence of oxygen.¹⁹ SAMs are stable in cell culture for periods of several days.²⁰ The optical properties of SAMs depend on the thickness of the underlying gold; SAMs supported on films of gold >1000 Å are opaque and reflective, and those supported on gold 100 Å in thickness are optically transparent.²¹ Even these thin films of gold are electrically conductive; this property permits a variety of strategies for non-invasive control of attached cells.²² These properties collectively make SAMs the best-available class of surfaces for studies in a variety of areas, and for studies of bio-interfacial science in particular.

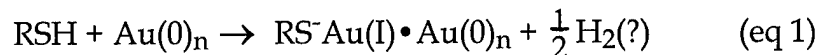
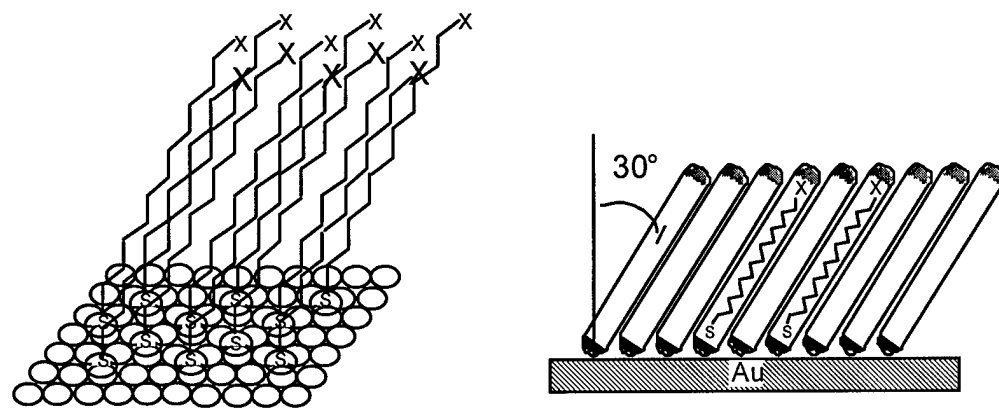


Figure 1. Representation of a self-assembled monolayer (SAM) of alkanethiolates on the surface of gold. (Left) The sulfur atoms of the alkanethiolates coordinate to the hollow three-fold sites of the gold (111) surface; the gold atoms (open circles) are arranged in a hexagonal relationship. The alkyl chains are close-packed and tilted approximately 30° from the normal to the surface. (Right) The properties of the SAM are controlled by changing the length of the alkyl chain and the terminal functional group X of the precursor alkanethiol.

In the first year of this program we developed surface chemistries that allow molecules to either be released from or immobilized to the substrate when an electrical potential is applied to the gold film.^{23,24} In the second year of the program, we demonstrated that the switching of the surfaces was compatible with mammalian cell culture, and that they could be used to influence the behavior of cells.²⁵ In the third year, we demonstrated a general method for

patterning cellular cocultures. This work has been published, and represents a key goal outlined in the original application.²⁶ During the final year of the program we have not been successful in patterning cocultures of fibroblasts and stromal cells and do not report on those experiments here. We have, though, directed our efforts at preparing a second type of electroactive substrate, one which could release adherent cells under electrical stimulation.

Active substrates that selectively release adherent cells have an important application to the study of heterotypic cell-cell interactions that were the goal of the original application. Whereas our initial efforts, which were directed at preparing substrates that could turn on the presentation of immobilized ligands, were important for patterning two cell types, this second class of electroactive substrate provides a new opportunity for mechanistic studies of the interactions of the two cell types. Specifically, the substrates allow the time over which both cells types are present to be controlled—by releasing one of the cell types to leave behind a patterned culture of the other cell type—and therefore can be used to determine the time over which heterotypic cell-cell communication is operative. Below we describe our work to demonstrate this second class of substrate and then conclude with an overview of the results of this project and the significance to cancer biology.

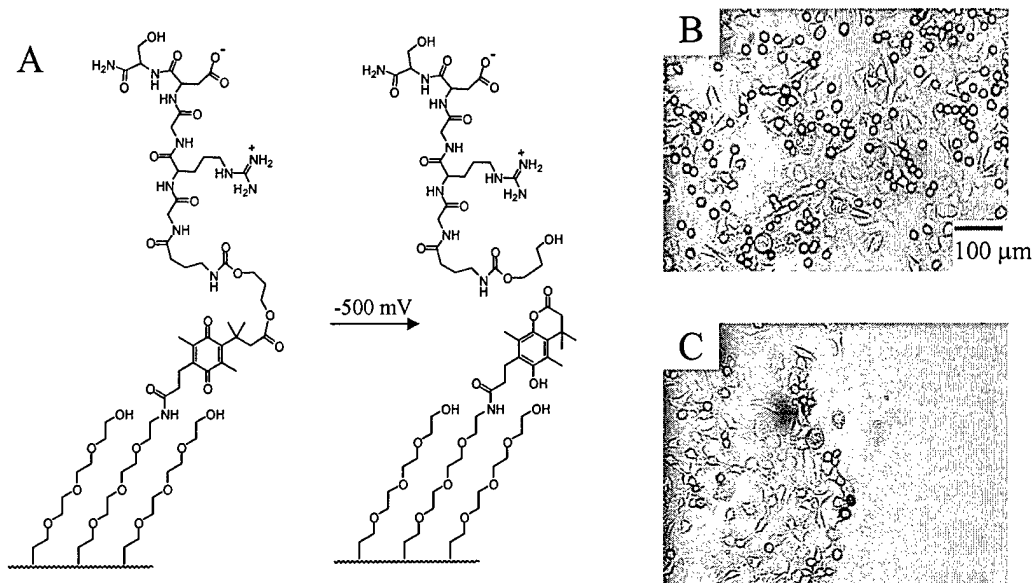


Figure 2. Electroactive monolayer that selectively releases cells. (A) The monolayer presents an Arg-Gly-Asp peptide ligand that is tethered by way of a quinone propionate ester. Electrical reduction of the substrate results in the hydroquinone, which rapidly cyclizes to release the peptide. (B) 3T3 Swiss fibroblast cells attached to a substrate presenting RGD peptides. The substrate was patterned such that the peptide ligands on the right half were tethered by way of the electroactive quinone group and those on the left half were linked directly to the monolayer. Application of the electrical potential results in release of ligands only on the right side, and hence of cells on that side.

Design of Electroactive Substrates that Release Ligands.

We had previously reported an electroactive substrate that could selectively release ligands on electrochemical stimulation.²⁴ The system was based on the electrochemical oxidation of a quinone-propionate ester and the subsequent intramolecular lactonization with release of the

ligand-bearing alkoxide side chain (Figure 2). In the last period we demonstrated that this active substrate was compatible with the conditions of cell culture and could be used to prepare substrates that selective release cells.²⁷ Figure 2 shows an example of cells that are attached to a substrate presenting the Arg-Gly-Asp peptide. After application of a reducing potential, the immobilized ligand is released, resulting in the detachment of fibroblast cells. This work is a first example of an active substrate that can selectively release adherent cells, but it has the disadvantage that the synthesis of the active alkanethiol is lengthy (approximately 25 steps) and hence not well-suited for biological experiments. Below we describe the recent development of a second generation system that is easier to prepare and operates by way of an oxidative (instead of a reductive) electrical stimulation. This latter point is significant because it is possible to prepare monolayers presenting two different ligands and trigger the release of either ligand independently of the other.

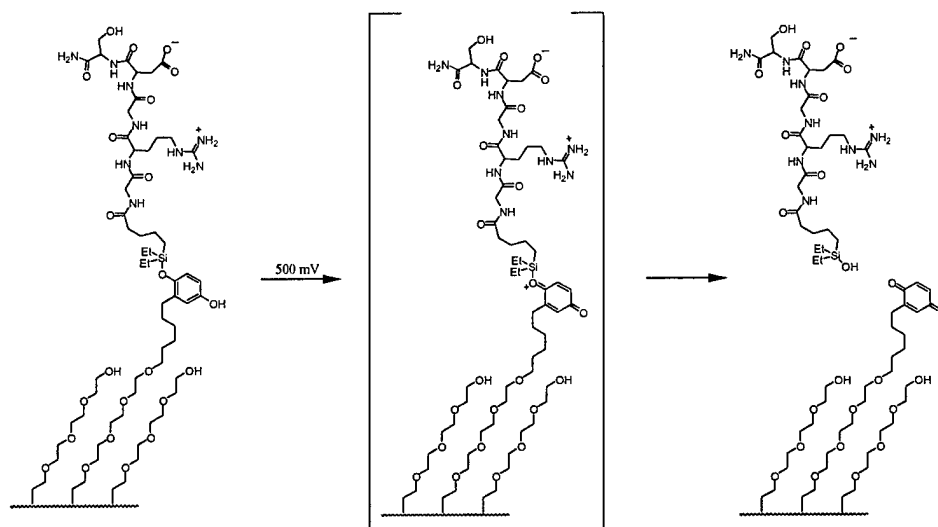


Figure 3. Design of a second generation substrate that releases ligands under electrical control. The peptide is linked directly to a hydroquinone moiety by way of a siloxane bond. Oxidation of this group results in hydrolysis of the Si-O bond and hence the selective release of the peptide.

Our second strategy relied on the oxidation of O-silylhydroquinones, which results in hydrolysis of the silyl-oxygen bond and release of the substituent. Figure 3 illustrates a monolayer that presents the Arg-Gly-Asp peptide by way of this electroactive linker. We first synthesized the required alkanethiol (Figure 4) and prepared monolayers containing 1% of this alkanethiol mixed with an alkanethiol terminated in the tri(ethylene glycol) group. The latter alkanethiol is important because it prevents the non-specific adsorption of proteins, and hence ensures that the interactions of cells with the substrate are mediated by the immobilized ligands alone. 3T3 Swiss fibroblast cells attached efficiently to these substrates. The cells adopted a well-spread morphology and had organized focal adhesions and cytoskeleton. Application of an electrical potential of 500 mV resulted in the release of immobilized peptide and hence of attached cells. A series of these experiments establishes that the substrates have the characteristics to be useful for a range of cell culture experiments; the substrates are stable in serum-containing medium; the substrates support biospecific cell adhesion; the substrates are compatible with optical and fluorescence microscopy; the ligands are efficiently and rapidly

released on application of short electrical potentials; the ligands can be patterned using several established methods.

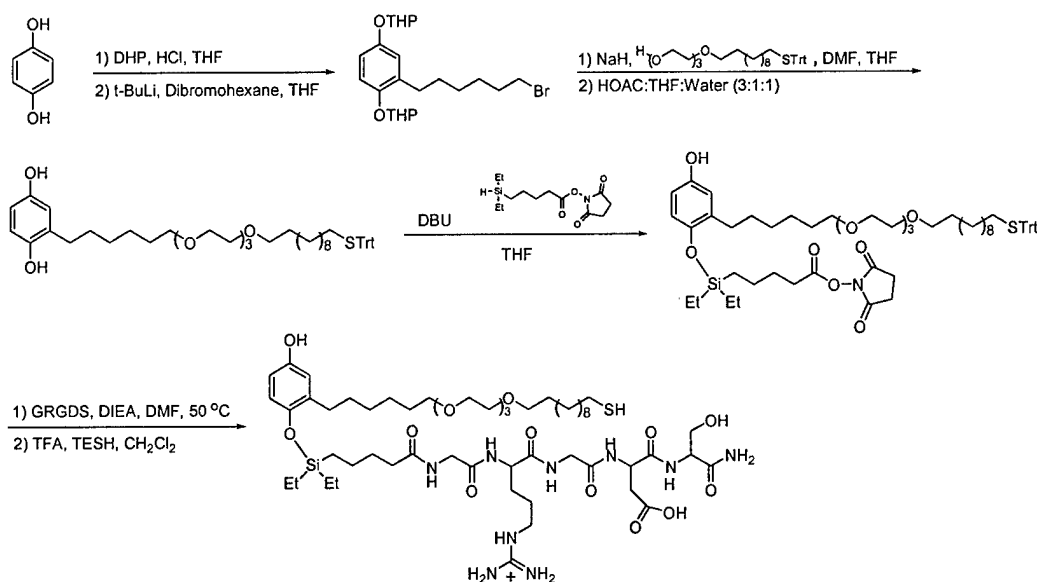


Figure 4. Synthesis of the alkanethiol that presents the Arg-Gly-Asp peptide tethered by way of the hydroquinone siloxane. Reagents are indicated with the transformation arrows.

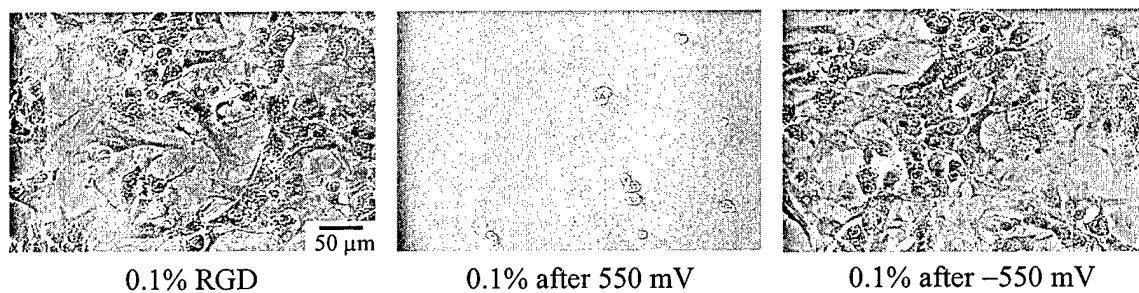


Figure 5. Optical micrographs of cell release from the electroactive substrate pictured in Figure 3. (Left) Cells attached and spread on a monolayer presenting the Arg-Gly-Asp peptide ligand at a density of 0.1%. (Right) After application of a potential of 550 mV, cells detached from the substrate, due to release of the peptide ligands. (Right) A control experiment showed that a negative electrical potential had no effect on cell adhesion or spreading.

Conclusions and Summary of Project

The results of this effort have been consistent with the goals stated in the original application. The most significant outcome is the demonstration of a new strategy—based on applications of surface engineering—for preparing dynamic substrates. These substrates—which can selectively immobilize or release ligands under electrical control—are unprecedented and will be of substantial value in a range of cell biology programs, and in particular for studies of heterotypic cell-cell interactions. These latter interactions are common to the growth and behaviors of cancer cells and surrounding normal cells, but have been difficult to study directly because of a lack of well-defined *in vitro* systems. The active substrates described here should address some of the prior limitations and lead to a better mechanistic understanding of metastatic processes.

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