



A Supramolecular System for the Electrochemically Controlled Release of Cells**

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The dynamic behavior of living mammalian cells is not only regulated by the cell itself but also influenced by a variety of external stimuli including cell–substrate interactions.^[1] The monitoring of cell behavior in the cellular environment has enormous implications for cell biology and regenerative medicine.^[2–4] Unfortunately, probing the response of cells to surrounding signals in real time is still a major challenge. Conventional methods, including flow cytometry, enzyme-linked immunosorbent assay, immunostaining, and polymerase chain reaction, are valuable but involve stepwise staining, washing, and manipulation before analysis. Other approaches involve staining with metabolically and chemically engineered probes. However, most of these assays measure markers under static conditions and fail to monitor what cells sense in real time, in a dynamic manner.

Alternatively, biomimetic substrates of which the surface properties can be dynamically controlled are used as tools to elucidate mechanistic information on cell fate such as signal transduction, adhesion, and migration.^[2] As redox reactions are noninvasive and give quantitative results, electrochemically programmable interfaces hold considerable promise as responsive substrates.^[5] To date, attempts to engineer the surface chemistry of substrates so that it can interface with cells with electrochemical control over cell fate decision have commonly involved covalent immobilization or physical entrapment strategies.^[6] The use of supramolecular chemistry is an attractive alternative way to achieve responsive biomimetic functions, as noncovalent interactions dominate the

formation of biological systems in nature and allow for reorganization to occur upon interaction with bioactive molecules.^[4c,7,8] Although there has been a number reports of these supramolecular systems and their novel responsive cellular applications, only a few have been shown to modulate cell–substrate interactions and only one example exists of a system that is responsive to electrochemical stimuli. In an elegant approach, Mrksich and co-workers showed how cell adhesion was restored upon positioning a carbonic anhydrase fusion protein containing an RGD motif with micromolar affinity between substrate-bound benzenesulfonamides and membrane-bound integrins.^[9] Teixeira and co-workers recently reported a route to achieve electroactive supramolecular control over stem cell differentiation; this control was achieved by modulating the availability of growth factors upon oxidation of a conjugated polymeric system.^[10]

However, synthetic supramolecular motifs employ well-established noncovalent interactions and their incorporation into molecular constructs has led to the formation of biological surfaces with novel properties.^[8b,11] Cucurbit[8]uril (CB[8]), a macrocyclic host molecule capable of binding two aromatic guest molecules simultaneously, is a suitable candidate for reversible binding where guest binding can be controlled by external conditions.^[12] As a result, a variety of biomimetic systems bearing this CB[8] host motif have already been produced.^[13] However, synthetic supramolecular host molecules have been scarcely used, thus far, for responsive cell adhesion and migration studies.^[14] Herein, we document a stable CB[8]-mediated system for the preparation of noncovalent cell adhesive surfaces that can be electrochemically controlled (Figure 1).

RGDS was functionalized with an N-terminal tryptophan-glycine-glycine (WGG) motif. The WGG tripeptide has been shown to bind as a ternary complex in the cavity of a viologen-CB[8] inclusion complex with an association constant of 10^5 M^{-1} .^[13a] Electroactive viologen was modified with an alkyl thiol group to achieve binding with a gold surface. As this ternary complex contains a peptide ligand that binds to integrin receptors and mediates cell adhesion, the immobilization of this complex yields a surface to which cells can attach efficiently. To confirm that CB[8]-mediated supramolecular complexation could occur in a culture medium that supports cell growth, the addition of the synthesized WGGRGDS peptide to a solution of viologen-CB[8] was monitored by UV/Vis absorption, emission, and ^1H NMR spectroscopy (see the Supporting Information, Figure S1). Broadening and upfield shifts of the aromatic signals in the ^1H NMR spectra of both viologen and tryptophan showed that both viologen and the indole moiety from the peptide

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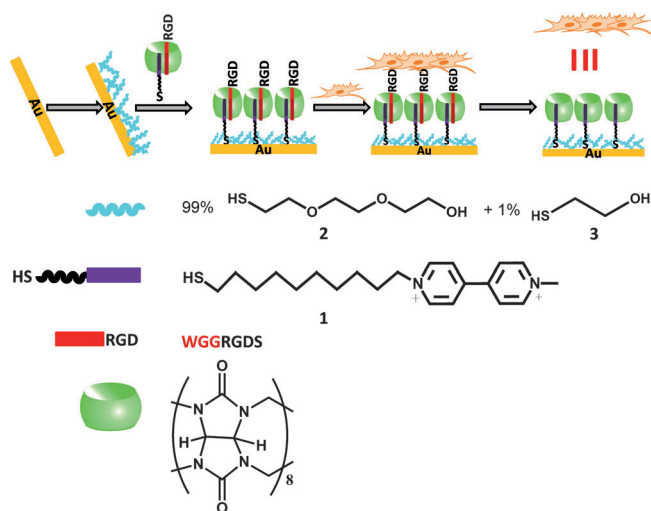


Figure 1. Strategy to assemble stable monolayers of the ternary complex that consists of WGGRGDS, CB[8], and **1**. The strategy starts with the immersion of slides (yellow) into a mixed solution of **2** and **3** (v/v: 99:1) to give the slides a cell-repellent nature. Subsequently, these cell-repellent slides are immersed into a solution of the ternary complex, thus resulting in specific insertion of the RGD complexes. After cell adhesion, electrochemical reduction of **1** leads to the release of the peptide and subsequent detachment of cells from the slides.

were bound in the cavity of CB[8]. Moreover, the appearance of a band at $\lambda = 430$ nm in the UV/Vis spectrum and the fact that the indole emission was quenched, as a result of formation of a charge-transfer complex between electron-donating indole and electron-accepting viologen in the CB[8] ternary complex, indicate that the peptide was bound as anticipated. Electrospray mass spectrometry (ESI-MS) further confirmed the existence of the ternary complex (see the Supporting Information, Figure S2). The thermodynamic stability of the RGD-conjugated ternary complex in cell culture medium was verified using isothermal calorimetry measurements (see the Supporting Information, Figure S3). A 1:1 binding stoichiometry was observed both in the case of the binding of viologen to CB[8] as well as in the case of subsequent binding of WGGRGDS to the 1:1 viologen-CB[8] complex with an association constant of $K_a = 3.6 \times 10^5 \text{ M}^{-1}$ and $K_a = 4.4 \times 10^4 \text{ M}^{-1}$, respectively, which are in good agreement with reported values for binding in aqueous solution of viologen, CB[8], and peptides.^[13]

Next, we fabricated monolayers that are modified with the RGD-conjugated ternary complexes on gold surfaces. These bioactive monolayers should meet key requirements such as: 1) the layers should be stable over the time course of the cell experiments to not only ensure high affinity complexation but also provide a well-packed monolayer,^[15] and 2) the substrates should be cell repellent prior to the installation of the ternary complex to the surface and upon release of the RGD peptide.^[6]

To this end, we adopted a strategy involving the insertion of the intact ternary complex into a cell-repellent monolayer (Figure 1).^[16] To analyse this process, attenuated total reflectance infrared spectra (ATR-IR) were recorded to directly compare the monolayers prior to and after insertion (Fig-

ure 2a). Although both monolayers of hexa- and tri-(ethylene)glycol (EG₆OCH₃ and EG₃OH) terminated thiols had excellent cell-repellent properties (see the Supporting Information, Figure S4), the insertion of the ternary complex

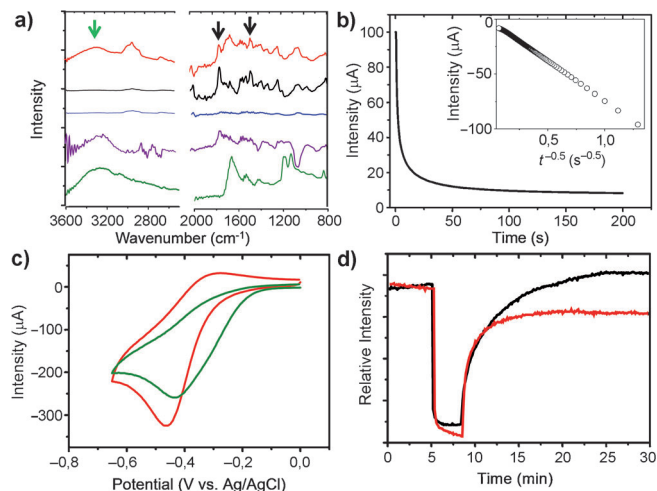


Figure 2. a) ATR-IR spectra of 1-CB[8]-WGGRGDS (red), 1-CB[8] (black), and MV-CB[8]-WGGRGDS (blue) on gold slides with a mixed monolayer of EG₃OH and EGOH, and MV-CB[8]-WGGRGDS (purple) and WGGRGDS (green) on gold slides without a mixed monolayer of EG₃OH and EGOH. Green arrows indicate the bands corresponding to the peptide and the black arrows indicate bands corresponding to CB[8]. b) Current transient after a potential step to -0.5 V (vs. Ag/AgCl) of a gold slide with 1-CB[8]-WGGRGDS. c) Cyclic voltammograms of the substrate modified with the ternary complex 1-CB[8]-WGGRGDS before (red) and after (green) the chronoamperometry experiment. d) SPR sensograms of 1-CB[8]-WGGRGDS (red) and 1-CB[8] (black) modified substrates. Normalized SPR intensities versus time upon electrochemical reduction of **1**. MV = methylviologen.

into these monolayers was unsuccessful. However, when monolayers of co-adsorbed EG₃OH (**2**) and 2-mercaptoethanol (EGOH, **3**) in a ratio of 99:1 (v/v) (see the Supporting Information for optimization details) were fabricated, the labile nature of the latter compound in the self-assembled monolayers (SAMs) of EG₃OH allowed the insertion of the RGD ternary complexes, of CB[8], WGGRGDS, and viologen (**1**) into these SAMs from solution.^[16] In this case, the typical IR bands of the peptide were observed at 3282 cm^{-1} and 1645 cm^{-1} as well as those of CB[8] at 1746 cm^{-1} and 1470 cm^{-1} (Figure 2a, red). Control experiments were performed using a ternary complex of CB[8], WGGRGDS, and methylviologen (MV), which lacks a thiol group. When this control ternary complex was assembled onto gold without the cell-repellent monolayer, the corresponding IR spectrum shows bands for both CB[8] and the peptide, thus indicating that they were adsorbed onto the gold surfaces (Figure 2a, purple). When we attempted to insert this control ternary complex into the mixed monolayer of EG₃OH and EGOH, IR bands indicative of neither CB[8] nor peptide were observed in the corresponding spectrum (Figure 2a, blue).

Taken together, these results indicate that the mixed ethylene glycol terminated monolayers prevent the unwanted electrostatic adsorption of CB[8] and the peptide to both the

underlying gold surface^[17,18] and the cell-repellent monolayer. Moreover, our strategy ensures the chemospecific and oriented insertion of the supramolecular complex into these cell-repellent monolayers through the thiol–gold interaction.

Surface plasmon resonance (SPR) spectroscopy with in situ cyclic voltammetry (CV) measurements together with several control experiments confirmed the incorporation of the ternary complex in our mixed monolayers of EG₃OH and EGOH. The monolayers only exhibited an electrochemical response corresponding to viologen when a thiolated methylviologen was used during the insertion step (see the Supporting Information, Figure S5). From the integrated areas of the reduction peak, the surface coverage of **1** in the case of the ternary complex was calculated to be $6.5 \times 10^{-11} \text{ mol cm}^{-2}$, thus indicating the presence of a tightly packed layer of RGD peptides on the surface. Furthermore, in our SPR set-up, chronoamperometry experiments were carried out by imposing a potential step to -0.5 V (vs. Ag/AgCl), which is cathodic to the first (reversible) one-electron reduction potential of -0.46 V (vs. Ag/AgCl), the potential at which viologen is reduced and thus the RGD ternary complex dissociates.^[19,20] As shown in Figure 2b the recorded current was found to decay continuously over time and a plot of its value versus $t^{-0.5}$ is linear, thus denoting a process where the current is diffusion limited (inset Figure 2b).^[21] Additionally, the release of the peptide was verified by the negative shift (30 mV) of the one-electron reduction potential of methylviologen, in agreement with literature data (Figure 2c).^[20] These results indicate that CB[8] remained on the surface through binding to **1**, whereas the peptide dissociates from the surface.

Furthermore, the change in reflectivity in the SPR sensorgrams prior to and after the chronoamperometric experiments was evaluated (Figure 2d). Upon applying a potential of -0.5 V at $t = 5 \text{ min}$, a sudden change in the SPR intensity was observed in the case of **1**·CB[8] monolayers (Figure 2d, black curve), thus indicating the formation of radical-cation viologen, which results in a broad plasmon resonance owing to strong optical absorption at the $\lambda = 633 \text{ nm}$ used in the SPR experiment.^[22] After removal of the negative potential at $t = 8.3 \text{ min}$, the initial SPR intensity was restored in 22 min, confirming that both dication viologen **1** and CB[8] remained on the surface. In contrast, when **1**·CB[8]·WGGRGDS monolayers underwent this electrochemical process, the SPR reflectivity was only partially recovered, thus indicating that the peptide was released from the substrate to solution during the electrochemical process.^[23]

With these results in hand, we next performed two cell experiments to demonstrate that these new dynamic supramolecular layers can be employed for the adhesion and release of cells. These experiments were performed on monolayers presenting the RGD ternary complexes using the above-mentioned assembly strategy. First, substrates were seeded with mouse myoblast C2C12 cells for 1 h in the cell culture medium. Limited cell adhesion was observed on surfaces that did not present the RGD peptides (Figure 3 and the Supporting Information, Figure S6), whereas on surfaces that were modified with the ternary RGD complex, cells efficiently attached and spread. After cells were cultured at 37°C for 1 h, an electrical potential of -0.5 V (versus Ag/

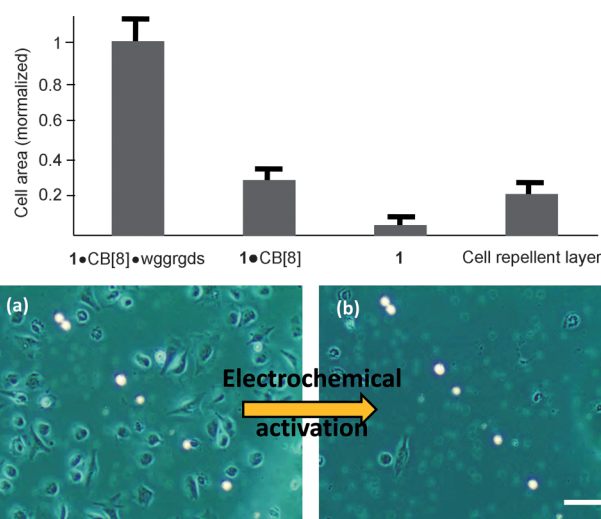


Figure 3. Normalized projected cell area (based on three independent cell seeding experiments) for the indicated functionalized slides after 1 h incubation of C2C12 cells (top). Bright field images of cells before (a) and after (b) electrochemical activation at -0.5 V (vs. Ag/AgCl) for 200 s. The white dots on the substrate serve as markers to indicate the same observation area. The scale bar is $100 \mu\text{m}$.

AgCl) was applied to the substrates at 37°C for 200 s. Over 90 % of the original adherent cells were removed from the surface by simple washing with saline and the remaining cells displayed a round morphology, indicative of poor adhesion to the surface (Figure 3b). Cell staining with a live/dead assay kit showed that cells had intact membranes and remained viable after a voltage pulse of -0.5 V for 200 s (see the Supporting Information, Figure S7). In another experiment human umbilical vein endothelial cells (hUVEC) were seeded and grown to confluence on the supramolecularly functionalized RGD monolayers (Figure 4a). In this case a wound-healing assay was performed in which the closure of a wound that was mechanically created was monitored.^[24] Figure 4b–g show the images of the wound closure within a 2 h interval. hUVECs on the RGD-functionalized substrates showed a faster recovery of the wound, that is, the migration speed, when compared to a control experiment on tissue culture plastic (see the Supporting Information, Figure S8). Taken together these results demonstrate that RGD coupled to the surface through the supramolecular CB linker is a stable surface for 10 h and displays specific cell adhesion with the ability of subsequent controlled electro-triggered release from surfaces.

To show that the developed supramolecular strategy is applicable to trigger cell adhesive response at the single cell

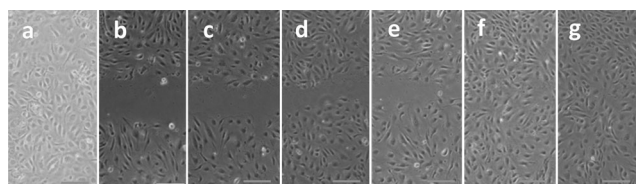


Figure 4. a) Confluent layer of hUVECs cells plated on slides modified with the ternary complex **1**·CB[8]·WGGRGDS and wound closure, imaged after 0, 2, 4, 6, 8, 10 h (b–g, respectively).

level, we next conducted cell experiments on an array of gold lines on glass substrates (Figure 5). Gold lines 5 μm wide and separated by 15 μm are individually addressable allowing for spatial and temporal control of the complexation of RGD peptides to the electrodes at the single cell level. After

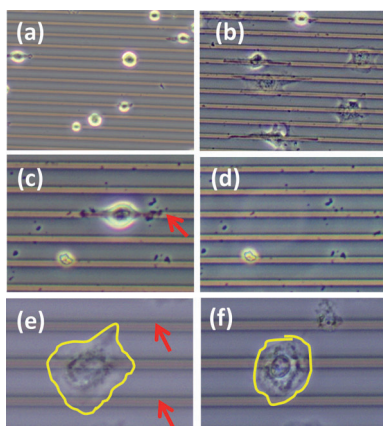


Figure 5. Bright field images of C2C12 cells cultured (30 min) on microelectrodes modified with a) only the cell-repellent layer and b,c,e) **1**-CB[8]-WGGRGDS complex. Images c) and e) are prior to electrochemical activation and d) and f) after electrochemical activation of the indicated areas in c) and e), respectively. The microelectrodes addressed with an electrochemical potential are indicated with red arrows. The width of the gold electrodes is 5 μm and the gaps between two electrodes are 15 μm .

covalently modifying the glass surfaces with an ethylene glycol polymer (see the Supporting Information), the substrates were immersed in a solution of EG₃OH/EGOH (99:1) and subsequently in a solution containing **1**-CB[8]-WGGRGDS. When C2C12 cells were plated for 20 min on a device, more than half of the cells attached to only one gold line while other cells connected different gold lines spanning across the intervening nonadhesive areas (Figure 5 b). Staining fixed cells with phalloidin showed that the localization of the actin filaments matched with the RGD-modified gold lines (see the Supporting Information, Figure S9). This result indicates that the RGD sequence specifically binds integrins, the major receptor for cell-extracellular matrix adhesion. Applying a voltage pulse to an individual supramolecularly RGD-modified gold line resulted in rapid desorption and detachment of the cells that resided on only that gold line (Figure 5 c and d). In the case a cells spanning between different gold lines (Figure 5 e), addressing a preselected gold line to release the RGD peptides triggered the detachment of the cell from only the target gold line (Figure 5 f). Additional control experiments, that is, cells plated on gold lines with either EG₃OH/EGOH (99:1), **1**, or **1**-CB[8] show rounded cell morphology (Figure 5, and the Supporting Information, Figure S10) and did not release after applying the same pulse.

In summary, a supramolecular methodology has been developed to immobilize RGD ligands onto gold slides to yield stable and specific interfaces for (single) cell experiments. Moreover, electrochemical activation results in dissociation of the supramolecular complex, release of the RGD

ligands, and detachment of cells from the surfaces. Access to tools to intervene with spatial and temporal resolution is important to study details of biological processes at the (sub)cellular level.^[25,26] The application of supramolecular chemistry provides the possibility to make fine adjustments in both thermodynamic and kinetic release aspects, potentially permits reversible associations,^[20] and provides entry to statistical variations of multiple orthogonal ligands. Currently, we are exploring the subsequent cell uptake of the supramolecularly released peptides to induce cell signalling.

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