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Transferrable Active Chemical Structures for Bottom-Up Heterogeneous Tissue Engineering

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14. ABSTRACT

Cell-cell interactions drive the organization of complex tissues. Studying these interactions has generally involved the use of either in vivo models or single-purpose microfluidic devices which cannot be reconfigured when experimental needs change. Here we show that graphene-based materials can be used as the basis for transferrable active chemical structures. We also demonstrate how these structures can be integrated biocompatibly with living tissue. In addition, we demonstrate passive structures that can be used to mask cells from various biochemical stimuli in a spatially pre-determined manner, and we extend the use of these passive structures to create multi-cell-type co-cultures.

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EXECUTIVE SUMMARY

Cell-cell interaction and communication is of central importance to the development and maintenance of complex multicellular organisms, as well as being critical to processes such as wound healing which are of interest to the DoD. Heretofore, the interactions between cells have been investigated using a number of different techniques. First, examination can occur on living organisms in vivo; second, global perturbations to groups of cells in vitro can be examined; and finally, microfluidic or microwell systems can be used to examine cell-cell interactions at the level of the individual cell. All three of these methods have advantages as well as drawbacks. In vivo research is generally highly restricted by ethics and can therefore be quite impractical to undertake. In addition, in vivo studies are more expensive than in vitro ones, as costs are incurred to keep and maintain live animals. Globally perturbative studies on cells are usually fast and inexpensive, but suffer from the drawback that spatially localized interactions between groups of cells often cannot be examined. Microfluidics and microwells are well-established alternative tools to examine these interactions, but microfluidic systems are generally purpose-built, and each new experiment requires the ex nihilo design and fabrication of a novel microfluidic system. This drawback adds complexity to experimental design in addition to being time- and cost-intensive. Thus new approaches are needed to design systems which can probe interactions between cells.

We have designed graphene-based materials which have the advantage of being patternable with chemically or electronically active structures using standard microfabrication techniques, while at the same time they are easily rendered biocompatible with standard off-the-shelf materials such as gelatin. They are simple to construct, modify, and deploy and very inexpensive to fabricate from scratch. To fabricate these materials, we have made advances in the chemistry of graphene-based materials. Additionally, we have extended the use of these materials not only to deliver active structures, but also to act as passive masks for controlling cell environments and enabling facile construction of multi-cell-type co-cultures.

This report presents research conducted by Keith Whitener (6177), Woo Kyung-Lee (6177), Dhanya Haridas (6177), Christopher So (6176), Saron Yoseph (6177), Matthew Thum (6124), Cy Tamanaha (6177), Xavier Langston (6177), and James Roberts (6177).

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TRANSFERRABLE ACTIVE CHEMICAL STRUCTURES FOR BOTTOM-UP HETEROGENEOUS TISSUE ENGINEERING

1. INTRODUCTION

1.1 Motivation and Background

The formation of complex tissues in multicellular biological systems is driven by biochemical and biophysical interactions between cells. This includes not only embryonic development, but wound healing and repair as well. Understanding cell-cell interactions is therefore of fundamental interest in cell biology and biomedical engineering.[1] A fine-grained understanding of cell communication and tissue morphogenesis processes of the kind that occur during wound healing might enable cell-by-cell wound healing which would result in less scarring, lower re-injury rates, and fewer chronic problems than the current state of the art.

There currently exists a number of methods for determining, measuring, and controlling these interactions, including synthetic biology,[2] microfluidics,[3] and microwell technologies.[4] However, drawbacks exist for each of these methods. Synthetic biology requires complex genetic manipulation of cells to obtain precisely tuned organisms on which to test hypotheses. Microfluidics and microwells are often bespoke, single-purpose systems which are not easily reconfigurable to probe a wide range of experiments. Microwells are also not generally designed to probe the interactions of cells within groups. The materials used in these experiments might also affect the outcome of certain experiments, as many cell types respond to both the chemical cues and physical (i.e., stiffness) cues to carry out their biochemical functions and adapt to their environment appropriately. A useful method would be highly conformal to the cells without perturbing them, and would furthermore be easily and inexpensively configurable to allow for rapid design and adaptation as experimental needs change.

We have developed a method for targeting cells that consists of highly flexible graphene-based membranes, which can be lithographed and patterned with structures which can be chemically or electronically active, and which can be used for sensing signals or for signal delivery. These structures are easily made using standard microfabrication techniques. We have rendered these membranes biocompatible, and we have shown that their presence is reversible and only weakly perturbative of normal cell viability. This technique will enable precise targeting of cells for evaluation of cell-cell interactions, while being easily reconfigurable and patternable.

1.2 Objective

The objective of this program is to construct graphene-based materials known as transferrable active chemical structures (TACS) and show that they can be modified in a biocompatible manner to interface polymeric and metallic structures with cells. Furthermore, this program aims to use TACS to probe and control intercellular interactions, which are fundamentally important to understanding and directing tissue regeneration and wound repair. The methods developed in this program serve as an alternative to existing microfluidic and synthetic biological approaches, both of which present issues in terms of complexity and lack of adaptability that the methods herein seek to overcome.

Manuscript approved July 27, 2022.

2. APPROACH

2.1 Graphene-enabled materials transfer

The enabling technology in this program was the discovery that chemically modified graphene (CMG) exhibits a much lower van der Waals graphene adhesion to its substrate than unmodified graphene.[5] As a result, CMG is easily delaminated from its substrate under very mild conditions, including by simple water dipping. In addition, the high mechanical strength of graphene-based materials enables patterning and lithography of structures and materials of various composition on CMG using standard microfabrication techniques. By coupling these ideas together, we can fabricate structures onto CMG and then detach those structures from the underlying substrate in a very mild water-based fashion. The detached structures can then be retrieved and relaminated onto an arbitrary substrate, effecting a transfer of surface structures and properties from one substrate to another.[6] Because the transfer is water-based and requires no harsh biocidal chemicals, one can transfer materials even to very fragile substrates, such as living cells.[7] We harnessed these ideas and observations to introduce TACS as a means to transfer interesting chemically active structures directly to biological materials for the purpose of understanding cell-cell interactions and the effects of the local microenvironment on cell behavior.

2.2 Ensuring biocompatibility of graphene-supported materials

Early in the program, we determined that direct transfer of graphene-based materials to cells without an interposing layer resulted in near-total cell death. This effect has been reported by a number of other researchers and is believed to be cause either by direct mechanical damage of the cells by the graphene nanosheets acting as nanotomes, or by elevated reactive oxygen species levels generated by the edges of the graphene sheets, which is known to induce apoptosis. Our observations point toward the former being the principal mechanism of cell death caused by graphene. We therefore began investigating ways to mitigate cell death while simultaneously ensuring close adhesion of the graphene-based structures to the cells. The experiments detailing these efforts are outlined in section 3.3.

2.3 Spatial control of cellular environments

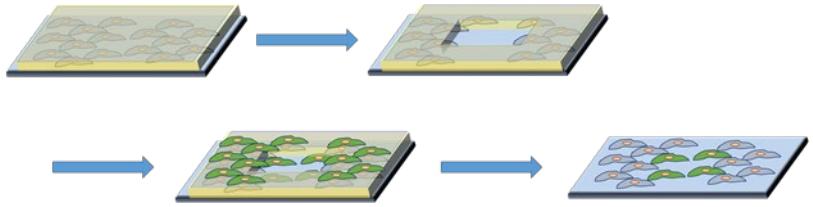

Fig. 1 — Schematic of graphene mask concept: a permeable pattern is etched into impermeable graphene to provide spatial control of biochemical delivery or cell coculture fabrication.

Ultimately, the approach developed in this program can aid in controlling and monitoring a cell's microenvironment. In addition to enabling transfer of active structures onto cells in a biocompatible manner, we have extended the technique described in the foregoing sections to passively control a cell's environment, using the concept of cell masking. We found that the membranes we prepare are highly impermeable to most substances. By patterning openings in the membranes, we can allow biochemicals to pass through at specific spatial locations. This technique enables us to mask some cells to a biochemical stimulus and expose other cells in a spatially dependent manner, allowing chemical control over a cell's environment. In addition to this cell masking approach for biochemistry, we have also used the masking concept to enable spatially precise co-culturing of multiple cell types in close proximity. We detail these efforts in section 3.4.

3. EXPERIMENTS

Sections 3.1-3.3 present an overview of experiments which have previously been published in peer-reviewed journals. The full details of these experiments are given in the relevant literature.

3.1 Hydrogenated graphene and reduced graphene oxide-based preparation and transfer of materials [7, 8]

Graphene and chemically modified graphene derivatives demonstrate remarkable mechanical strength-to-weight properties. Thus, single layer hydrogenated graphene (SLHG), while only ~1 nm in thickness, can support materials such as patterned polymers and metals that are 1000 times thicker than the underlying graphene sheet. We demonstrated that SLHG could delaminate from its substrate while carrying patterned gold and polymers. We also built simple electronic devices on SLHG, including a graphene nanoribbon field effect transistor (gFET) and a polymer-based ammonia gas sensor. We showed that these devices were operational both before and after transfer to a number of different surfaces, including SiO_2 and polyethylene.

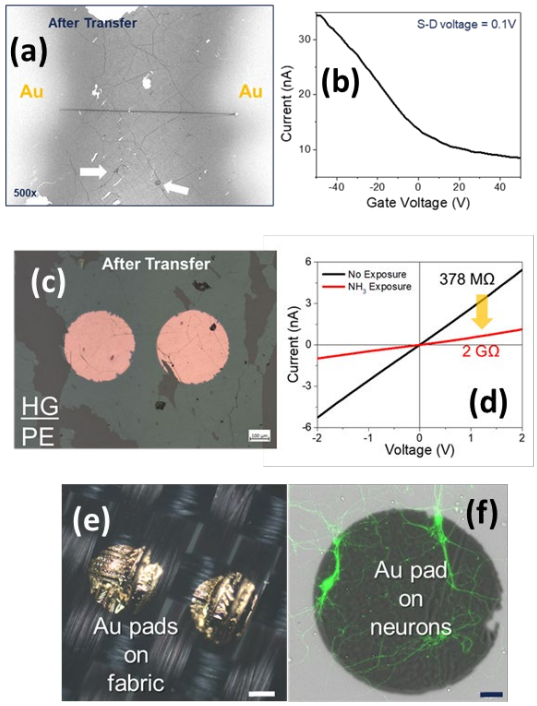

Fig. 2 — SLHG based transfer of TACS. (a) Graphene-based field effect transistor (gFET) after transfer. (b) IV curve demonstrating field effect behavior of gFET. (c) Polymer-based ammonia gas sensor after transfer. (d) IV curve demonstrating gas sensing properties. (e) Gold pads transferred onto rough fabric surface. (f) Gold pads transferred to living neurons. Adapted with permission from Ref. 7. Copyright 2019 John Wiley & Sons Inc.

While SLHG has an extremely large strength to thickness ratio, in absolute terms, it is only about 1 nm thick. As a result, the devices built and transferred on SLHG show a significant degree of degradation and issues with reproducibility become apparent. This is mainly due to the fact that the transfer process itself is not particularly gentle and induces holes, tears, and other defects in the graphene sheet. To circumvent this problem, we designed and fabricated TACS based on reduced graphene oxide (RGO) films. These films are multilayer and multiflake graphene, so they have a lower strength to thickness ratio. However, they are significantly thicker than SLHG (typically 5-50 nm, depending on preparation conditions), which allows higher reproducibility and ease of transfer without significantly thickening the

transfer membrane. The preparation of these RGO membranes was accompanied by an evaluation of their chemical and physical properties, including functionalization and permeability. These developments guided and enabled further experiments described below.

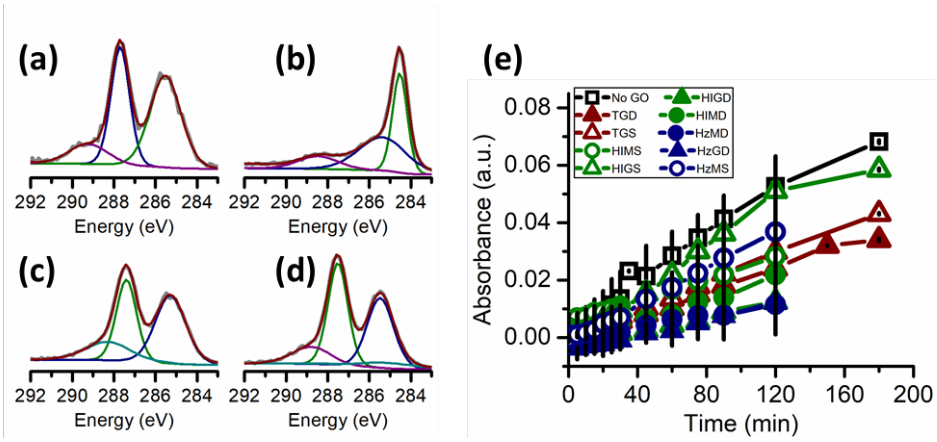

Fig. 3 — Chemical and physical characterization of RGO membranes prepared under different conditions. (a) C1s peak of X-ray photoelectron spectrum (XPS) for untreated graphene oxide. (b) C1s peak of XPS of thermally reduced RGO. (c) C1s peak of XPS of hydrogen iodide reduced RGO. (d) C1s peak of XPS of hydrazine reduced RGO. (e) Permeability of RGO membranes prepared under different conditions. Reprinted with permission from Ref. 8. Copyright 2021 Elsevier.

One other major advantage of using RGO over SLHG was that RGO can be obtained via either thermal or chemical reduction of graphene oxide. This is important because it greatly expands the number of substrates that can be used for preparing the RGO-based TACS. Before the use of chemical reduction to effect transferability of the films, the only substrates that were usable for preparation of RGO were ones which could withstand being rapidly heated to over 200°C. Thus, most flexible or polymer substrates were not useful from a transferability standpoint. However, our findings that chemically reduced RGO recapitulated the transfer properties of thermally reduced RGO allowed us to expand the number of viable substrates to include low-melting polymers and flexible materials, which can be manipulated more readily than stiff glass or non-polymeric substrates.

3.2 Graphene- and gelatin-assisted transferrable lithography [9]

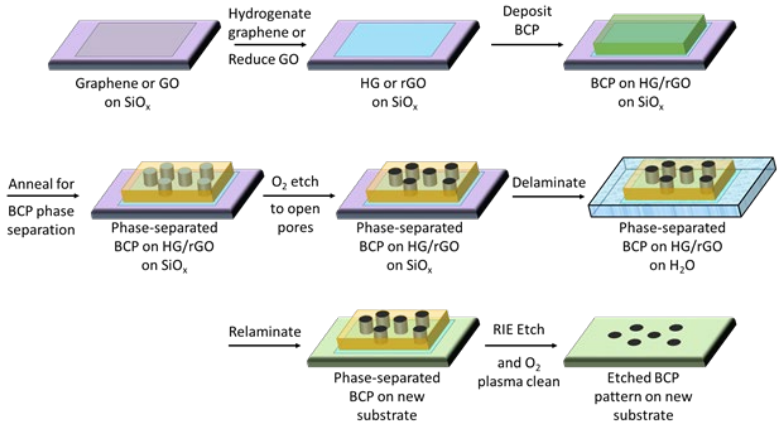

Fig. 4 — Schematic diagram of transferrable BCP lithography technique. Reprinted from Ref. 9 under the terms of Creative Commons Attribution 4.0 License (CC-BY). Copyright 2021 IOP Publishing Ltd.

The transferability of materials on graphene was extended to block copolymers and photopolymerizable materials. This technique enables pre-patterning of micro- and nanoscale features into a polymer mask, whereupon solution-based and reactive ion-based lithography techniques can be applied to etch these features into an arbitrary substrate. For instance, polystyrene-based block copolymer lithography can be performed in this fashion directly on a polystyrene substrate, which would be impossible using a standard solvent annealing process. In addition, we showed that different block copolymer orientations can be achieved and patterned on a single wafer, and block copolymer patterns can be stacked to form hierarchical structures, none of which is possible using standard block copolymer lithography. Thus the fact that the block copolymers can be developed first and transferred afterwards opens up a wide range of possibilities in nanoscale lithography and fabrication.

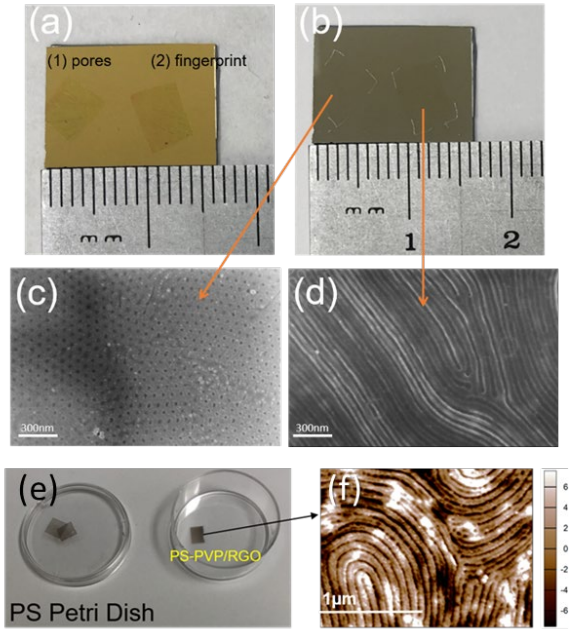

Fig. 5 — Transferrable block copolymer lithography. (a) Transferred developed block copolymer/graphene oxide mask on SiN substrate before etching. (b) Mask and substrate after etching. (c) SEM of etched nanopores (~30 nm diameter). (d) SEM of etched nanogrooves on the same SiN surface (~30 nm pitch). (e) Transferred developed polystyrene-based masks on polystyrene dish. (f) AFM of etched polystyrene. Reprinted from Ref. 9 under the terms of Creative Commons Attribution 4.0 License (CC-BY). Copyright 2021 IOP Publishing Ltd.

In addition to block copolymers, we examined the use of SU-8, a common photoresist used in microfabrication, in conjunction with graphene-based and non-graphene-based transfer. SU-8 was spincoated onto RGO and gelatin surfaces and UV exposed under a shadow mask, followed by development in solvent. The SU-8 on RGO can be transferred in the standard way onto arbitrary substrates, including curved tubes and living matter. We found that one drawback of this method was that the RGO transfer substrate was quite difficult to etch away, even using high energy density oxygen plasma. This creates an issue when attempting to use the SU-8 as a mask on these new substrates. As a mitigation of this problem we found that SU-8 can be patterned and developed directly on a gelatin substrate with very good fidelity. The gelatin can then be dissolved in hot water and the SU-8 floated on the water's surface to dry before transferring it to an arbitrary substrate. This method eliminates the need for etching RGO windows into the SU-8 mask. We used these methods to control cell microenvironments, described below in section 3.4.

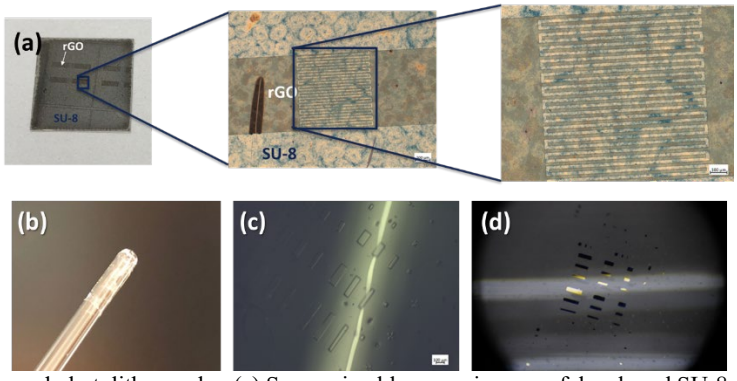

Fig. 6 — Transferrable SU-8 based photolithography. (a) Successive blown up images of developed SU-8 patterns on RGO, ready for transfer. (b) SU-8 mask transferred onto a curved surface NMR tube. (c) Optical microscope image of SU-8 features as small as 2 μm on curved surface. (d) Evaporated gold on curved surface after mask removal.

3.3 Biocompatible graphene- and gelatin-assisted transfer of printed structures to cells [8]

As mentioned in section 2.2, directly draping graphene over cells with intimate contact leads to nearly complete cell death after only a few minutes. In addition, once the graphene is in contact with the cell, it is irreversibly bound to the cell, so that even if there were a way to keep the cell alive while in contact with the graphene, it would be impossible to remove the graphene for subsequent access to the cell.

This irreversibility actually stems from how well the surface energies of the cell and the graphene material match. The cell surface is naturally very hydrophilic, whereas SLHG and RGO are both quite hydrophobic. The mismatch between the graphene and the cell surface renders adhesion between the two surfaces very weak, and the graphene delaminates from the cell surface immediately upon reintroduction of the cells to liquid media. There are three possibilities to obtain a stronger interaction between the surfaces. First, we could attempt to make the cell more hydrophobic. The main way to do this is to dry the cell slightly. This method allows the graphene to adhere strongly to the cell, but it also leads to near universal cell death. Second, the graphene could be chemically modified to include more hydrophilic functional groups. However, this approach generally causes the delamination of the graphene from the initial (usually glass or SiO_x/Si) substrate to fail. One could potentially combine the two approaches to create a Janus-type membrane with one hydrophilic and one hydrophobic side, but this approach would ultimately still encounter biocompatibility issues with direct contact between the graphene and the cells.

We employed a different approach, which was to use an intermediary layer between the cells and the graphene. We evaluated several different types of intermediary layers using substances known to be biocompatible. These substances included alginate, agarose, zein, and specially designed peptides. These peptides were designed with one end as a cell linker (RGD motif) and the other end as a graphene binding motif taken from the literature.[10] However, we found that none of these substances had the combination of biocompatibility and ability to adhere strongly to cells that we needed.

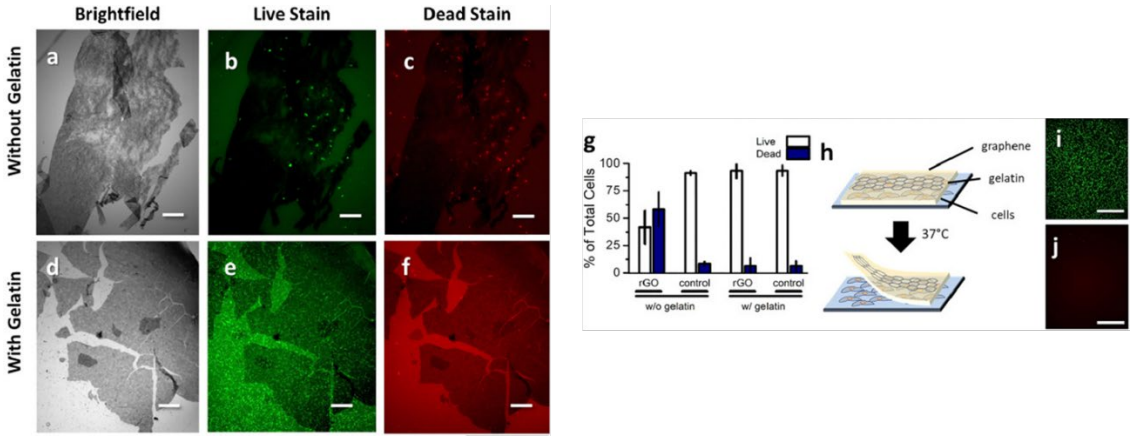

Fig. 7 — Biocompatibility of graphene/gelatin combination. (a)-(c) Brightfield, live stain, and dead stain microscopy images of graphene in direct contact with cells, without gelatin. (d)-(f) Brightfield, live stain, and dead stain microscopy images of graphene on cells with gelatin intermediary layer. (g) Live-dead cell count for graphene on cells with and without gelatin. (h) Schematic of gelatin/graphene removal at 37°C. (i)-(j) Live and dead stain microscopy images after graphene/gelatin removal. Reprinted with permission from Ref. 8. Copyright 2021 Elsevier.

We found that gelatin acts as an ideal intermediary layer between the graphene and the cells. Gelatin is an inexpensive and easily obtainable hydrogel manufactured from the acid hydrolysis of collagen. It is extensively used for its biocompatibility. Gelatin melts into a viscous liquid above 37°C and dissolves easily in water and ethanol. Importantly for our purposes, the hydrophobicity of gelatin changes based on water content. Thus, gelatin will easily coat a highly hydrophilic surface such as a layer of cells, but its air-facing layer will quickly dry out to form a very hydrophobic surface, which interfaces readily with hydrophobic graphene derivatives. In addition, the bulk of the gelatin remains highly hydrated, allowing the cells to exchange nutrients and waste with their environment. This feature, along with the physical separation of the cells from the graphene, dramatically increases cell viability.

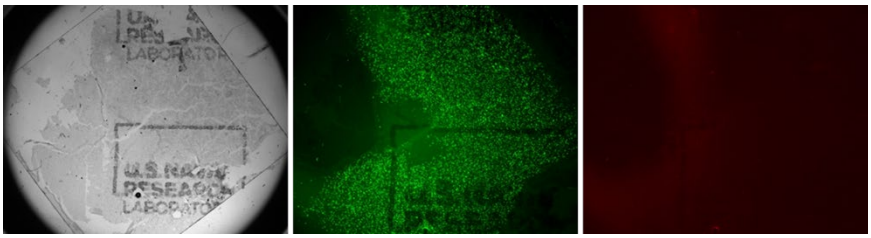

Fig. 8 — Brightfield and live/dead stain microscopy of printed silver structures on chemically reduced RGO transferred to cells coated with gelatin for biocompatibility. Reprinted with permission from Ref. 8. Copyright 2021 Elsevier.

The development of a biocompatible protocol for integrating TACS with cells allowed us to design, print, and transfer structures onto cells. As mentioned in section 3.1, we developed a chemical reduction method to produce transferrable RGO, and coupling that method with gelatin-derived biocompatibility enabled us to produce transferrable flexible structures on RGO while maintaining cell viability.

3.4 Graphene and SU-8 masks toward spatially well-defined cell environments and co-cultures

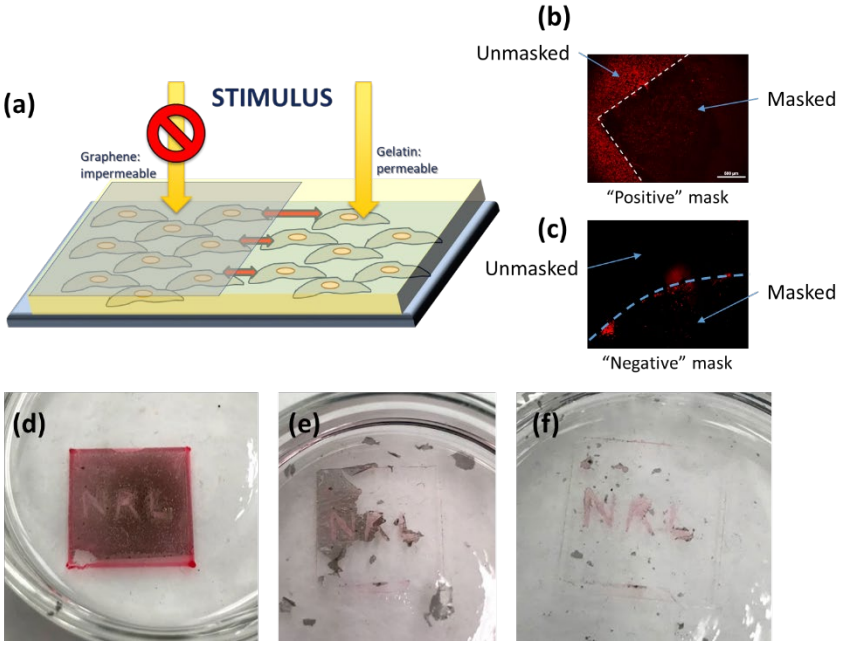

Fig. 9 — Cell masks for biochemical delivery. (a) Schematic of cell mask concept. (b) "Positive" mask, where the stimulus is added to the media and the mask prevents the cells under it from receiving the stimulus. (c) "Negative" mask, where the stimulus is added to the mask and the unmasked cells receive less of the stimulus. (d) Mask on dyed gelatin being prepared for photocrosslinking. (e) After photocrosslinking the gelatin with sodium persulfate and $[Ru(bpy)_3]^{2+}$, the unmasked areas are crosslinked, while the masked areas remain ordinary gelatin. By subjecting the gelatin to 37°C water, the masked gelatin dissolves, removing the graphene mask at the same time. (f) After the masked gelatin has dissolved, only the heat-resistant crosslinked gelatin in the unmasked region remains.

The impermeability of the graphene membrane enables another capability with TACS; namely, that of cell masks. Shapes or openings patterned into the graphene membrane are permeable windows between the cell media and the cells themselves, whereas the areas where graphene remains are much slower to exchange media between the cells and the large media reservoir. This results in stimuli added to the media being preferentially uptaken by unmasked cells and not by masked cells. We show in Figure 9b an example of this spatially selective delivery, in this case of a cell dye (Rose Bengal).

We provide an additional proof of concept in Figures 9d-f. Here we have applied a photocrosslinking chemistry to gelatin under a graphene mask (RGO with the letters "NRL" scratched out of it). The chemistry is a photooxidation of tyrosine groups present in the gelatin to form covalent crosslinked bonds. The oxidizer (sodium persulfate) and the photosensitizer $[Ru(bpy)_3]^{2+}$ are added to the media above the gelatin, and visible light is supplied from below. The impermeable graphene prevents the gelatin underneath from being exposed to the crosslinking chemicals, whereas the openings in the graphene allow direct contact between the gelatin and the crosslinking chemistry. When light is applied for 1 min, only the gelatin that was exposed to the chemistry crosslinks. This validates the mask idea as a proof of concept. We are currently further applying this concept beyond graphene, by adapting the SU-8 photopatterning transferability to the cell mask idea. We are able to pattern SU-8 masks and transfer them onto cells without using graphene at all, by doing all of the development of the mask directly on gelatin, which can then be removed in hot water.

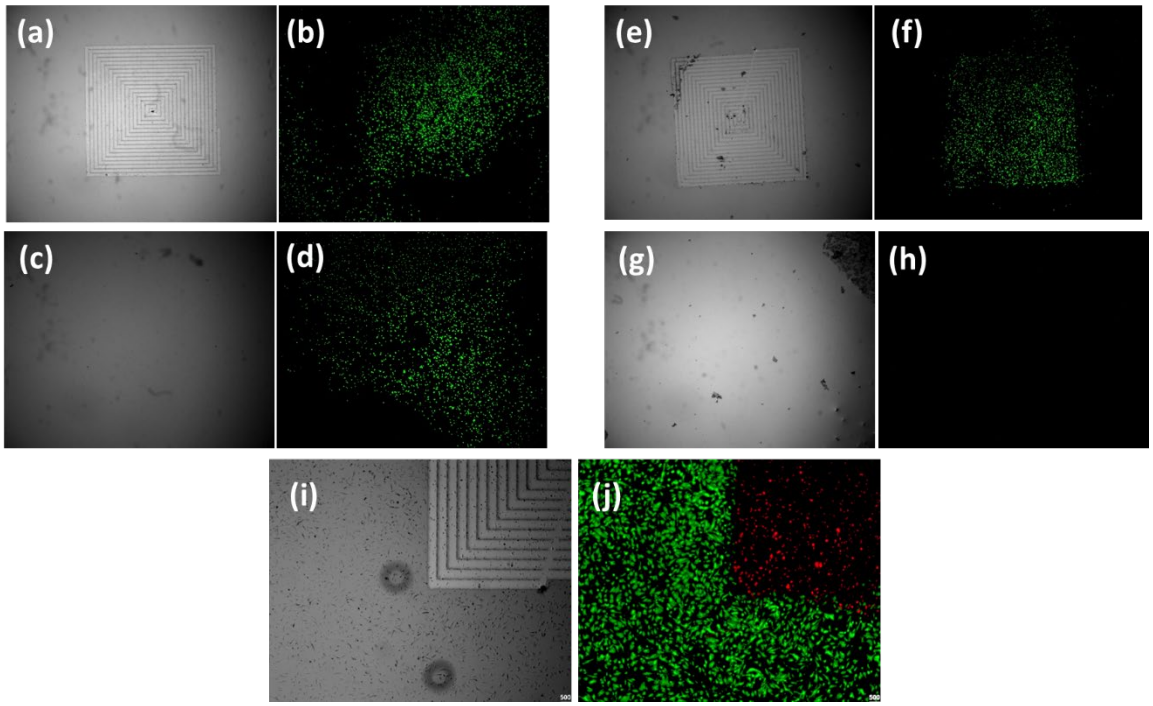

Fig. 10 — Cell masks for fibroblast/MSC co-cultures. (a, b) Brightfield and live stain of laser-ablated spot on ITO slide coated with gelatin only and reseeded with MSCs. (c, d) Brightfield and live stain away from laser-ablated spot on ITO slide, coated with gelatin only and reseeded with MSCs. (e, f) Brightfield and live stain of laser ablated spot on ITO slide coated with gelatin/RGO and reseeded with MSCs. (g, h) Brightfield and live stain away from laser-ablated spot on ITO slide, coated with gelatin/RGO and reseeded with MSCs. (i, j) Brightfield and composite image of CellTracker green and red fluorescent stains at laser ablated spot on ITO slide with gelatin/GO. Green are previously seeded fibroblasts and red are newly seeded MSCs.

Finally, we are using the graphene mask concept in conjunction with the BioLP (Biological Laser Printer), an NRL-developed technology based on laser induced forward transfer,[11] whose initial purpose was to precisely transfer cell types onto specific locations. It does this by laser ablating an indium tin oxide (ITO)-coated slide on which a cell culture has been grown. Those cells are pushed off the slide onto a new substrate. We adopt a simplified workflow by working with the ITO slide directly. In a typical experiment, we grow human dermal fibroblasts on the ITO slide, and then coat the cells with gelatin and a continuous piece of RGO membrane. We then ablate a specific geometry from the ITO slide using the BioLP laser. Afterward, we reseed a new group of cells, namely mesenchymal stem cells (MSCs), on top of the graphene membrane. Once the cells have settled somewhat, we can remove the mask and gelatin at 37°C. The cells on the mask (MSCs) are removed, but the cells under the mask (fibroblasts) and in the ablated area (MSCs) remain, giving easy access to spatially precise cell co-cultures using multiple cell types in close contact. Figure 1 provided a schematic of the process, and experimental results are shown in Figure 10.

4. CONCLUSIONS

Based upon the above data, we have shown that the TACS approach to cell monitoring and cell-cell interaction is a promising one. We have demonstrated that graphene-based materials have desirable chemical and physical properties which allow for active chemical and electronic structures to be built on them, and which enable transfer of these structures mildly to a wide variety of substrates, including living cells. Graphene-based materials on their own are generally not biocompatible, but we have shown that they can be made biocompatible by incorporation of biopolymers such as gelatin, which also facilitates adhesion between cells and graphene. We have shown that graphene materials can be utilized not only as active

materials, but also passive materials interacting with cells in the form of graphene masks, which enable precise spatial control of cells' microenvironments. In addition, these graphene masks, in conjunction with BioLP technology, enable facile design and fabrication of spatially precise multi-cell-type co-cultures.

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