

Biocompatible, Robust Free-Standing Paper Composed of a TWEEN/Graphene Composite

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Nonspecific binding (NSB), a random adsorption of biocomponents such as proteins and bacteria on noncomplementary materials, is one of the biggest problems in biological applications including biosensors, protein chips, surgical instruments, drug delivery, and biomedicine. Polyoxyethylene sorbitan laurate (TWEEN), a commercially available chemical with aliphatic ester chains, has shown promise as a medical material and in overcoming problems associated with NSB.^[1–4] However, stability during solution-based processing and uniformity of the materials that have TWEEN coating on flat substrates or nanomaterials using the self-assembled-monolayer (SAM) method has been an important issue. Further, biocompatible materials with high strength are important for several medical applications including stents, nail implants, and strong invasive instruments. Here, we present the production of a free-standing “paperlike” material composed of TWEEN and reduced graphene oxide (RGO) platelets and obtained by simple filtration of a homogeneous aqueous colloidal suspension of TWEEN/RGO hybrid. The “TWEEN paper” was highly stable in water without leakage of TWEEN and is compliant and sufficiently robust to be handled by hand without breaking. Furthermore, the TWEEN paper was noncytotoxic to three mammalian cell lines and biocompatible, inhibiting nonspecific binding of Gram-positive bacteria.^[5] In contrast, RGO paper without TWEEN showed nonspecific bacterial binding.

TWEEN is composed of three chemical parts (Fig. 1a): aliphatic ester chains that can prevent NSB of biomolecules, three-terminal hydroxyl groups that are hydrophilic and can be chemically modified for further applications, and an aliphatic chain that can

easily be adsorbed on a hydrophobic surface by noncovalent interaction. Protein microarrays on flat substrates with SAM of TWEEN^[4] and highly sensitive biosensors,^[1–3] built using field-effect transistor (FET) behavior of individual carbon nanotube (CNT) strands coated with TWEEN, have demonstrated that TWEEN can be effectively used to overcome NSB.

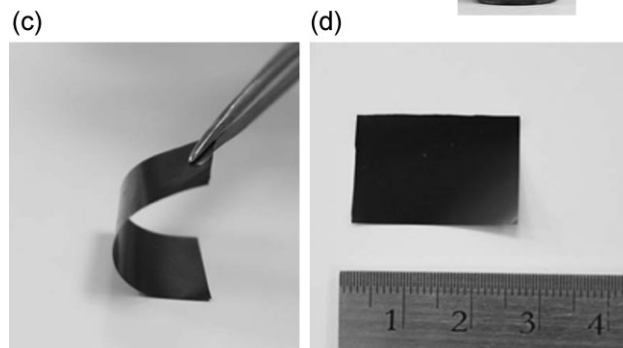
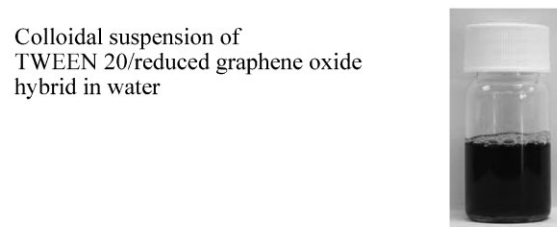
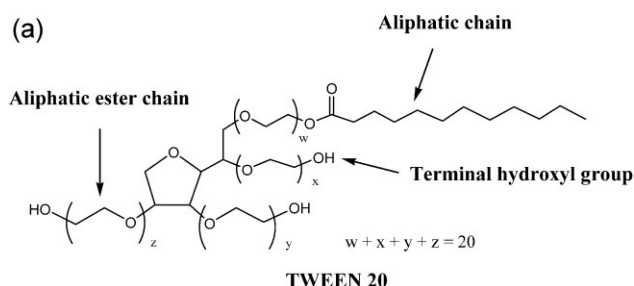


Figure 1. a) The chemical structure of TWEEN 20. b) Reaction scheme for the production of an aqueous colloidal suspension (photo, bottom right) of TWEEN/RGO-hybrid particles. c,d) Photos of a TWEEN paper sample (scale in (d), cm).

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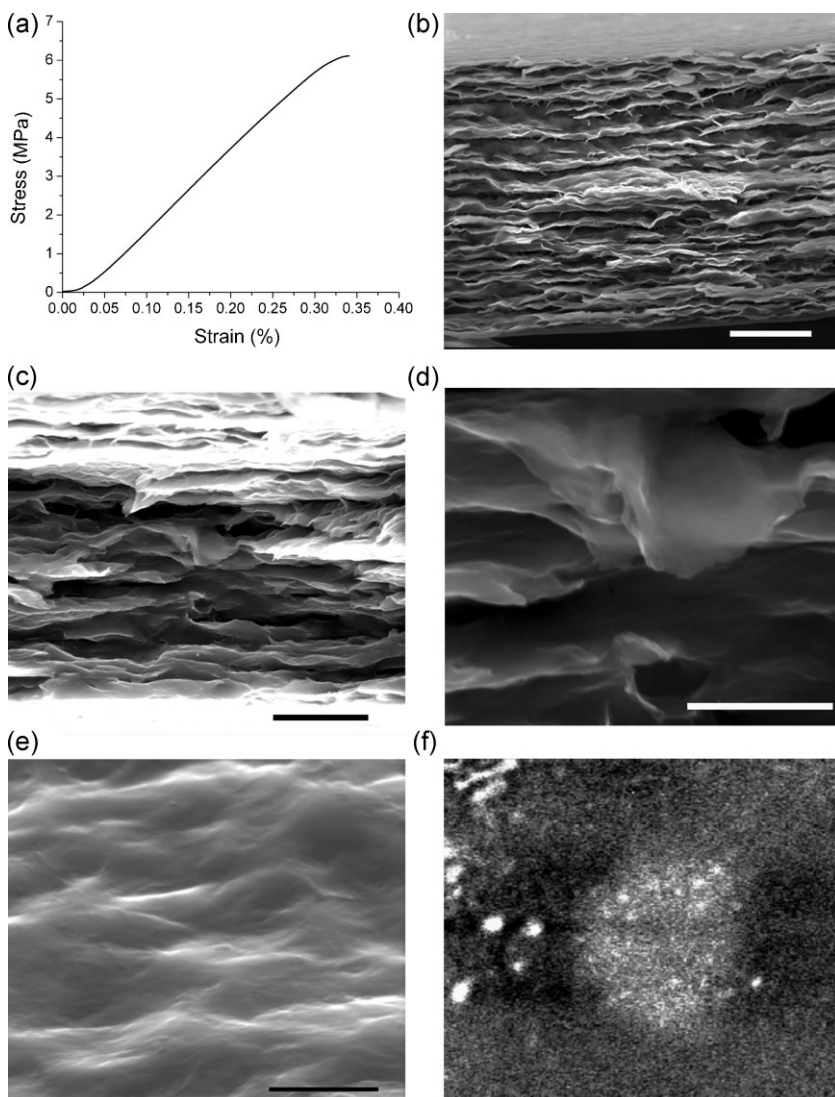


Figure 2. Mechanical properties of a TWEEN paper sample and images of SEM and Raman spectroscopy. a) A stress–strain curve of the paper sample by tensile test. b–d) SEM images of a cross-section of broken sample by tensile test (b) (scale bar, 10 μm) and a sample fractured by tweezers at room temperature (c,d) (scale bars, 5 μm (c) and 2 μm (d)). e,f) Surface images of the paper sample; an SEM image (e) (top view at an angle; scale bar, 2 μm), and a mapping image by micro-Raman spectroscopy (f) (scale bar, 6 μm).

Graphene, an atom-thick layer composed of sp^2 carbons, has recently gained interest owing to its outstanding electrical, mechanical, and thermal properties.^[6–8] Bioapplications using graphene sheets have been suggested for fundamental research as well as practical applications.^[9–11] Hydrophobic graphene sheets, which are exfoliated from graphite/graphite-derivatives or produced by reduction of graphene oxide, have shown noncovalent adsorption by surfactants, resulting in the generation of their homogeneous colloidal suspensions.^[12–14] Consequently, we thought that TWEEN might easily adsorb on the basal planes of graphene sheets. The high specific surface area of graphene (the calculated value is $2630 \text{ m}^2 \text{ g}^{-1}$, which includes both surfaces) would also benefit its applications as a highly sensitive sensor for gas or biomolecules.

TWEEN paper was the same as that of the sample before further soaking in the measuring range of our balance (measurable to 0.1 mg), so TWEEN molecules are not leaked from the paper sample in water, or the leak is negligible.

The weight ratio of chemically reduced graphene oxide (without TWEEN, other conditions were the same as that for production of TWEEN/RGO hybrid describe above) relative to starting GO was $\sim 65\%$, as measured for several separate reactions in our laboratory. That is, 19–20 mg of chemically reduced graphene oxide has usually been produced by reduction of 30 mg of starting GO. The weight ratio of RGO in the TWEEN paper relative to the starting GO would be similar to that of RGO in the system without TWEEN, meaning that $\sim 51 \text{ mg}$ of paper, which is produced from 30 mg of starting GO,

Graphite oxide (GO) was synthesized from natural graphite (SP-1, Bay Carbon, MI, USA) by the modified Hummers method.^[15] A homogeneous black suspension of TWEEN 20/RGO hybrid with no particles visible to the naked eye has been produced by chemical reduction of an aqueous colloidal suspension of graphene oxide and TWEEN using hydrazine monohydrate (Fig. 1b, see the Supporting Information (SI) for details). On the other hand, reduction of aqueous graphene oxide suspension using hydrazine monohydrate without TWEEN produced black agglomerates that could not be redispersed by sonication or stirring.^[16] This indicates that TWEEN plays a role as a surfactant for helping dispersion of RGO sheets in water. We confirmed that the chemical structure of TWEEN was not changed by the reaction with hydrazine monohydrate (see the SI). The presence of individual RGO sheets in the hybrid was confirmed by non-contact-mode atomic force microscopy (AFM; see the SI, Fig. S2).

Shiny black and compliant TWEEN-paper samples (Fig. 1c,d) composed of the TWEEN/RGO particles were fabricated by filtration of the homogeneous aqueous suspension of TWEEN/RGO hybrid (see the SI for further details). Using the homogeneous suspension provides a very promising method for generating uniform performance of such paper-like materials. After harsh rinsing with water and subsequent air-drying (as described in SI), the weight of the resulting TWEEN paper was higher than that of the starting GO (e.g., 51.1 mg of TWEEN paper from 30.0 mg of starting GO material and 17.6 mg from 12.0 mg). The leakage of TWEEN, which is adsorbed on the hydrophobic surfaces, from materials is an important issue in terms of stability.^[4,17] To check the stability of the TWEEN paper in water, the washed and air-dried paper sample was further soaked for 4 days in water and then air-dried for 3 days (see the SI). The weight of the resulting

contains 19–20 mg (37–39 wt%) of RGO. Thus, ~31–32 mg (61–63 wt%) in the TWEEN paper (~51 mg) should be contributed by TWEEN 20 and possibly trapped solvents in the paper samples. The thermogravimetric analysis (TGA) curve (see the SI, Fig. S3) of the air-dried TWEEN paper sample showed almost no weight loss before 100 °C. A weight loss (~48 wt% relative to the starting material) from 100 to 300 °C and a smaller weight loss (~14 wt% relative to the starting material) from 300 to 550 °C could be caused by loss of CO, CO₂, and H₂O by the decomposition of C–C and C–O bonds in TWEEN 20 and/or labile oxygen functional groups of RGO^[15,16] and, possibly, by evaporation of water molecules trapped in the paper sample. The remaining material (~38 wt% of the starting mass) at 550 °C in the TGA curve is close to that (37–39 wt%, calculated by the ratio of weight of the RGO to the starting material, see above) of RGO in the TWEEN paper sample.

The TWEEN paper samples were sufficiently robust to be handled by hands and tweezers without breaking. We have measured the mechanical properties of the air-dried paper materials weighing ~51 mg by tensile testing (five samples; Fig. 2a and the SI, Table S2). The TWEEN papers were brittle, similar to other graphene-based paper materials^[8,11,18] and had a modulus in the range of 1.2–2.3 GPa in the “linear region” (this region is almost linear, thus here we refer to it as the linear region). The tensile strength of the TWEEN papers was 3.4–6.2 MPa and their strain was 0.2–0.4% (see the SI, Table S2).

While the surface of the RGO paperlike sample is electrically conductive,^[19] the surface of TWEEN paper is electrically insulating. This could be caused by complete coating of electrically insulating TWEEN molecules on RGO platelets. Scanning electron microscopy (SEM) images of the cross-section of such an air-dried TWEEN paper samples (broken by the tensile test, Fig. 2b, and separately fractured by tweezers, Fig. 2c and 2d, at room temperature) exhibited overall a layered structure of TWEEN/RGO hybrid (Fig. 2b,c). However, determining the structural nature between TWEEN matrices and RGO platelets could be an interesting challenge for the future. The existence of thin “graphene sheets” in the composites was frequently found by SEM (Fig. 2d). The thickness of a sample broken by the tensile test was ~30 μm (Fig. 2b). The Raman spectrum of the surface of the air-dried paper sample showed two broad peaks at around 1345 and 1580 cm⁻¹. These two peaks would be contributed mainly by a D-band and a G-band from embedded RGO sheets, respectively.^[19,20] The Raman spectrum of free TWEEN 20 exhibited two weak broad peaks, only, at around 1290 and 1475 cm⁻¹. Although the surface of the TWEEN paper sample (Fig. 2e) was slightly rough, the mapping images of the surface at around 1345 cm⁻¹ and separately 1580 cm⁻¹ showed uniform dispersion of RGO platelets in the TWEEN matrices at the surface of the paper sample (Fig. 2f).

Cytotoxicity analysis of the TWEEN paper was carried out by investigating cell growth

and proliferation^[11] on the surface of the papers. Three cell lines representing a broad range of characteristics in cell growth were employed: i) Vero cells (African green monkey kidney cells)—robust cells with high propensity of growth and proliferation, ii) embryonic bovine (EB) cells—primary culture with a limited lifespan resembling the cells in vivo, and iii) Crandell–Rees feline kidney cells (CRFK)—finicky, delicate cells with relatively low growth rate and proliferation. After washing the cells with phosphate buffer saline (PBS), followed by their incubation on the TWEEN paper in the presence of a fresh culture media for 48 h (see the SI for details), the three cell lines were analyzed for growth, structural properties, and vitality. A rough estimate of cell death is inferred from the transformation of the cell's native structure into a spherical shape and the floating of cells detached from the surface of the paper upon washing. The confirmatory live–dead test (calcein and propidium iodide staining) on the cells growing on the TWEEN paper confirmed its noncytotoxicity, with >90% of the cells being alive after 48 h of exposure to the paper and forming a confluent monolayer on its surface (Fig. 3), indicating cell vitality during growth. Although TWEEN is known to degrade the cell membranes, the noncytotoxicity of TWEEN paper could be attributed to its firm incorporation into the RGO paper. These cytotoxicity tests (see the SI) were also conducted on the RGO paper without TWEEN, produced with the method explained in a previous publication.^[19] As expected, the papers showed a consistent cell growth and proliferation with the cells retaining their native shape, and forming a confluent monolayer as observed by the live–dead test. The observed noncytotoxicity of the RGO paper is consistent with an earlier report by Chen et al., where mouse fibroblast cells were used.^[11] Further, the two papers (TWEEN, and RGO paper) were nonhemolytic in blood-agar experiments (see the SI, Fig. S1). Blood cells in the agar exposed to the papers were found to be stable without any sign of lysis (and discoloration).

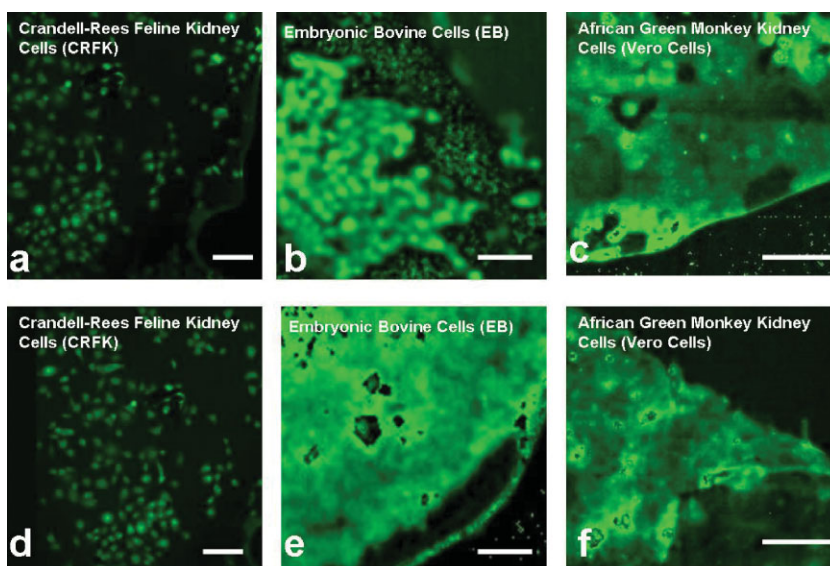


Figure 3. Cytotoxicity test for the TWEEN paper and the pristine RGO paper. Composite confocal microscopy images of the CRFK (a,d), the primary EB (b,e) and the Vero cells (c,f), grown for 48 h on TWEEN-paper (a–c) and RGO-paper (d–f) substrates, respectively, and subsequently stained with calcein (green) and propidium iodide (red) as a part of the standard live–dead test (scale bars, 20 μm).

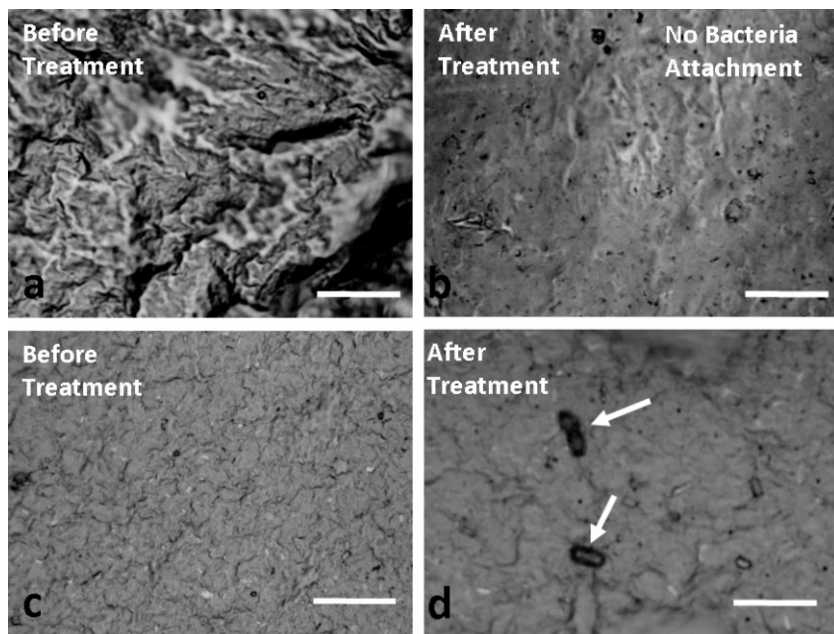


Figure 4. a,b) Optical microscopy image of the TWEEN paper before and after the treatment with a DI-water suspension of the mature *Bacillus cereus* cells. The TWEEN paper shows no bacterial attachment. c,d) Optical microscopy image of the RGO paper before and after the bacterial treatment. The RGO paper shows bacterial attachment (marked by arrows). All scale bars are 10 μm .

To study their NSB to microbes, the two papers were tested for bacterial interfacing. While the TWEEN paper inhibited NSB of Gram-positive bacteria, the RGO paper exhibited bacterial binding. Here, the papers were submerged in a suspension of washed *Bacillus cereus* cells in deionized (DI) water for 1 min and for 10 h (Fig. 4, and see the SI; Fig. S4), rinsed with excess DI water for 10 s, dried in a slow jet of nitrogen and observed under an optical microscope (see the SI for details). No bacterial cells attached on the surface of the TWEEN paper^[3,4] (Fig. 4). To further confirm the absence of bacteria, the exposed papers were seeded in separate nutrient-broth media (10 mL) and incubated at 37 °C in an incubator-shaker for 16 h, followed by centrifugation (11000 g (13 000 rpm) for 10 min) of the media-solution. No bacterial growth was observed, as deduced from the absence of pellet formation after centrifugation. In contrast, the RGO paper exhibited bacterial attachment and subsequent bacterial growth in a sterile media solution. The difference in the cell adhesion properties between the bacterial cells and the mammalian cells for the TWEEN paper can be attributed to the dissimilarities between the Gram-positive-bacteria cell wall (thick, rigid peptidoglycan layer with teichoic acid moieties) and the cell membrane of the mammalian cells (lipid membrane with cell adhesion molecules (CAMs), including immunoglobulins, integrins, cadherins, selectins, and/or lymphocyte receptors).^[21–23] Further, after reducing the density of the CAMs on the Vero cells via trypsinization (trypsin proteolysis) of the CAMs, followed by a short exposure (1 min) to the papers, no cell adhesion was detected on the TWEEN and the RGO paper (see the SI, Fig. S5). In conclusion, the TWEEN paper reduces the NSB of the bioentities, including the Gram-positive bacterial cells; while

it allows adhesion and proliferation of the mammalian cells, which use CAMs to stick to the TWEEN paper.^[3,4] More work is necessary to confirm the bacterial cytotoxicity of TWEEN paper and to thoroughly characterize its interaction with the cell's surface proteins, teichoic acids, and peptidoglycan membrane.

In summary, we have fabricated a strong and biocompatible free-standing paper composed of TWEEN 20 and chemically reduced graphene oxide and demonstrated that the paper exhibits excellent stability in water and is compliant and is robust enough to be handled by hand without breaking. The TWEEN paper and the RGO paper were noncytotoxic to three mammalian cell lines, while only the TWEEN paper inhibited the NSB of bacterial cells. These results show that the macroscopic TWEEN-based paper materials and its derivatives can potentially function as effective components for medical applications including transplant devices, invasive instruments, and implants.

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