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Collagen Coating Promotes Biocompatibility of Semiconductor Nanoparticles in Stratified LBL Films

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ABSTRACT

Nanostructured thin films fabricated from semiconductor nanoparticles (NPs) are of great interest for biomedical applications, but NP materials based on heavy metals can be cytotoxic. In this work, the preparation of semiconductor NPs followed the protocol of layer-by-layer (LBL) assembly, which alleviates this problem. Collagen/poly(acrylic acid) bilayers were added to CdTe/polycation LBL films to produce porous collagen bilayers. Such stratified multilayer systems showed successful cell attachment and survival while native NP films were strongly cytotoxic.

Introduction. Polymer/inorganic nanocomposites hold substantial promise for the production of novel materials in which optical, electrical, magnetic, and catalytic properties of inorganic nanostructures are combined with optical, electrical, and mechanical properties of macromolecules. The mosaic of chemical and physical characteristics available for permutations from both classes of compounds provides a versatile platform for materials designed for different purposes. Numerous prototype devices designed to utilize the unique material properties of nanoparticles (NPs) have been developed for primary applications in advanced optical, electrical, chemical, and mechanical systems.[1] The potential integration of these materials with living systems could lead to the development of promising technology for diagnostic and therapeutic applications in medicine and biology. A new class of biomaterials can be created in which the unique properties of nanoparticles are combined with biological functionalities. For type II−VI semiconductor and similar NPs, the progress in this field was limited in part due to their cytotoxicity. Coating NPs with SiO_2 and organic polymers can render them sufficiently biocompatible to allow ex-vivo and animal studies.[2−5] Uncoated particles remain difficult to use in contact with biological tissues, however, they are essential when utilizing electrical and/or distance-dependent collective optical properties.

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A critical emerging issue in the use of NPs concerns functionalization or modification of NPs to impart biocompatibility, targeting specificity, and assimilation of biological properties to NPs. These issues are essential if NPs are to be used for fabrication of in vivo diagnostic and therapeutic devices. Manipulating the biological and inorganic interface will eventually allow us to develop biocompatible nanoscale prosthetics. Such an interface may also include a "buffer zone" that is required to make a transition from the nanocomposites to living tissues. It will allow these materials to both coexist and function. A new fabrication technique known as LBL assembly used for the preparation of nanoparticle/polymer composites can indeed smooth property gradients between disparate materials.[6−8] This technique appears to be convenient for constructing functional interfaces because (1) layers can be assembled with both inorganic NPs and biomolecules and (2) the composition of the film can be easily changed from layer to layer to impart the desired properties. The aim of this work is to demonstrate that a simple, multilayer composite can provide certain features of biocompatibility to LBL layers of uncoated CdTe nanoparticles, which are otherwise strongly cytotoxic.

Experimental Section. *Chemicals.* All the chemicals, unless otherwise specified, were purchased from Aldrich (Milwaukee, WI) and were used without further purification. Collagen type IV (acid soluble, from human placenta) was purchased from Sigma-Aldrich (St. Lois, MO). Ultrapure water rated at 18 MΩ/cm was used for all experiments and for washing. The pH of solutions was adjusted with diluted HCl or NaOH. All experiments were performed under atmospheric conditions, except synthesis of semiconductor nanocrystals.

Procedures. Aqueous colloidal solutions of thioglycerol-capped CdTe nanocrystals were prepared as described previously.[9] Before the assembly, the substrates were subjected to an extensive cleaning procedure. Glass and silica wafers were cleaned in freshly prepared "piranha" solution (2:1 concentrated 98%H_2SO_4/30%H_2O_2; DANGEROUS in contact with organic matter) for 5 min then rinsed extensively with water and finally dried under a stream of nitrogen. Petri dishes were used as received.

The LBL assembly was started with absorption of positively charged poly(dimethyldiallylammonium) chloride (PDDA), Mw = 400 000−500 000 on a glass, silicon, or plastic substrate, which is negatively charged due to the existence of native thin oxide layer on the surface. The sequence of operations resulting in the production of semiconductor NP films was the following: (1) dipping of the substrate into a solution of PDDA (0.5%, pH = 3) for 10 min, (2) rinsing with water for 1 min; (3) dipping into the dispersion of CdTe nanocrystals for 20 min; (4) rinsing with water again for 1 min. On each exposed surface, such a procedure resulted in a "bilayer" consisting of a polymer/NP composite. The cycle can be repeated as many times as necessary to obtain a multilayer film of desirable thickness.

The biocompatible coating of collagen on the surface of assembled LBL films of NPs was built in the same cyclic manner. After a 1 min wash of the substrate in deionized water, the CdTe multilayer film with a PDDA-primed surface was dipped into a 0.5 wt % solution of poly(acrylic acid), Mw = 400 000−500 000 (PAA) at pH 4, which changes the surface charge to negative. Following the same washing procedure, the substrate was exposed to positively charged 0.1% solution of collagen type IV at pH 4 for 20 min and rinsed with water again. The further growth of PAA/collagen bilayers was accomplished by the repetition of the same cycle of immersion into the solution of PAA, rinsing, immersion into the collagen solution, and rinsing. This procedure results in the deposition of a film with a layer sequence of (PDDA/CdTe/)$_n$PDDA(/PAA/collagen)$_m$, where n and m are the number of the corresponding deposition cycles. After the final assembly cycle, the substrate was dried with a stream of nitrogen.

For cell culture studies, the films were deposited on the bottom of a tissue culture-treated plastic dish, which served as the LBL substrate. In this case, the polyelectrolyte, semiconductor nanocrystals, collagen solutions, and water for rinsing were poured into the Petri dish and remained there for the specified length of time. Some cell culture experiments used thin films deposited on a 25 mm^2 diameter microscope cover glass. The cells were seeded on the LBL films 3−5 days after the deposition. During this time they were stored in the refrigerator at 4 °C. Prior to seeding the surface was treated with as an antiseptic agent, 70% ethanol, and air-dried.

Muscle myoblast cells (C2C12) were obtained from American Type Culture Collection (ATCC, Manassas, VA). Rat phaeochromocytoma cells (PC12), a well-characterized neuronal cell model, were a gift from Dr. Giulio Taglialatela. C2C12 cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) containing 15% fetal bovine serum (FBS, Hyclone), 2 μg/mL streptomycin, and 200 U/mL penicillin. PC12 cells were cultured in DMEM with 10% FBS. The cells were removed from culture substrates with a brief trypsin/EDTA treatment and centrifuged. Before seeding, polystyrene Petri dishes or coverglasses with deposited LBL layers were sterilized with ethanol and air-dried in a sterile hood. Cells were suspended in fresh medium and were seeded on the substrates and allowed to adhere in a 37 °C 5% CO_2/95% air environment for specified times (incubator, NuAir Inc., Plymouth, MN). For LDH assay, C2C12 cells were maintained for 1 day and PC12 cells were maintained for 3 days after seeding onto the sample dish. After this period, cell culture medium was changed to the assay medium. After 6 h, absorbance for LDH activity at 490 nm was measured.

Cytotoxicity was determined by assaying the activity of lactate dehydrogenase (LDH) in the media (Roche Molecular Biochemicals, Takara Bio Inc.). LDH is an intracellular enzyme that is released into the media upon cell death and subsequent lysis of the cell. The activity of the enzyme is determined with a colorimetric assay where an optical density value is directly proportional to the number of dead cells. As it is released into the cell culture supernatant, LDH participates in a coupled reaction converting a yellow tetrazolium salt to red formazan. The amount of enzyme

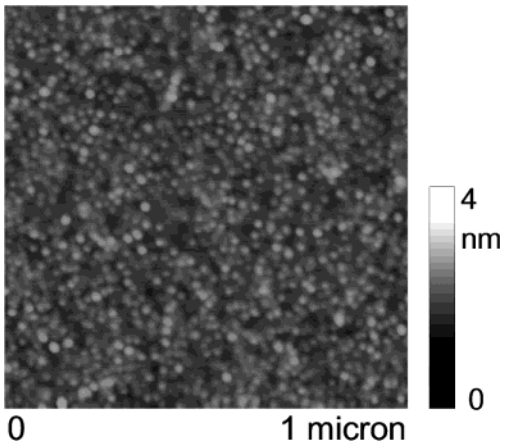

Figure 1. AFM image of PDDA/CdTe bilayer.

activity correlates to the number of damaged cells. The entire procedure, including cell growth and color measurement, may be performed in a single multiwell plate.

The analysis on soluble Cd was performed by Accurate Labs and Training Center, Stillwater, OK.

Instrumentation. Surface imaging of nanoparticles layers was performed by Nanoscope III (Digital Instruments/Veeco, Santa Barbara, CA). Atomic force microscopy (AFM) images were obtained in tapping mode with standard Si/N tips.

Ellipsometric measurements were made with an AutoEL MS ellipsometer from Rudolph Research Corp. (Flanders, NJ). The measurements were performed using a 632.8 nm line of He/Ne laser incident upon the sample at 70°. The DafIBM program supplied by Rudolph Technologies was employed to determine film thickness.

UV-vis spectra were taken using a HP8453A diode array Hewlett-Packard spectrophotometer. Attached cells were imaged using a Microflex UFX-DX inverted microscope (Nikon USA, Melville, NY) with 100× and 200× magnification.

Results and Discussion. LBL assembly is actively being utilized in the biomedical field in the production of micro- and nanocapsules for drug delivery.[10] The effects of contact between polyelectrolyte capsules and tissues have not yet been investigated. The first studies investigating the direct contact of solid LBL biomaterials and living cells appeared only recently,[11−18] and most of these works were concerned with polyelectrolyte−polyelectrolyte multilayers. Two other studies involve particles of calcium phosphate[12] and titanium dioxide nanoshells[14] where parent materials are known for their intrinsically high biocompatibility. However, all of these studies were concerned the materials that lack the physical and optical properties of luminescence, noticeable electrical conductivity, or magnetism found in NPs from less biocompatible materials, such as II−VI semiconductors.

Preparation and Structure. The LBL deposition of negatively charged thioglycerol stabilized semiconductor CdTe NPs with positively charged poly(diallyldimethylammonium chloride), PDDA, as a partner polyelectrolyte was described in several previous publications.[8,19,20] The structure of these films can be visualized by atomic force microscopy in a single $(PDDA/CdTe)_1$ bilayer (Figure 1). The NPs are closely

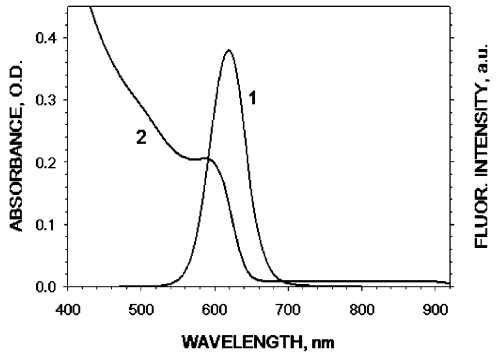

Figure 2. Photoluminescence (1) and absorption (2) spectra of $(PDDA/CdTe)_{10}$ LBL assembled film.

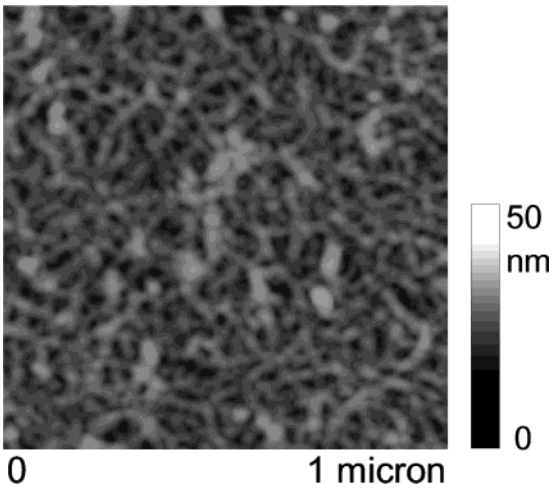

Figure 3. AFM image of PDDA/PAA/collagen assembly.

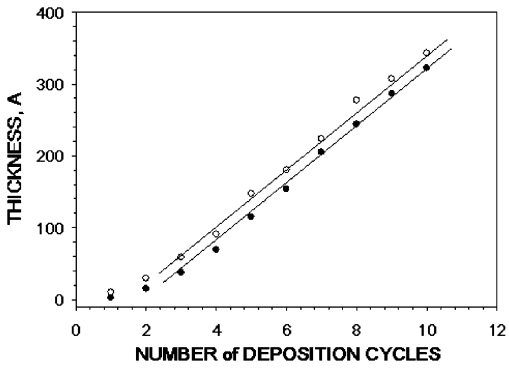

Figure 4. Ellipsometric measurements of the thickness of $PDDA(PAA/collagen)_n$ multilayers, $n = 1-10$, for collagen (closed circles) and PAA (open circles) layers vs. the number of adsorption cycles, *n*.

packed, forming a uniform coating of the substrate; the average height of surface features is ∼1.5 nm. Importantly, the films reveal strong luminescence with a quantum yield of 16% after the deposition (Figure 2, trace 1). The wavelength of the luminescence is 620 nm, which is quite convenient for monitoring the optical processes through scattering media such as tissue, cell culture, or polymeric cell support due to low attenuation of light by scattering in this spectral region.[8,9] The films also have the strong

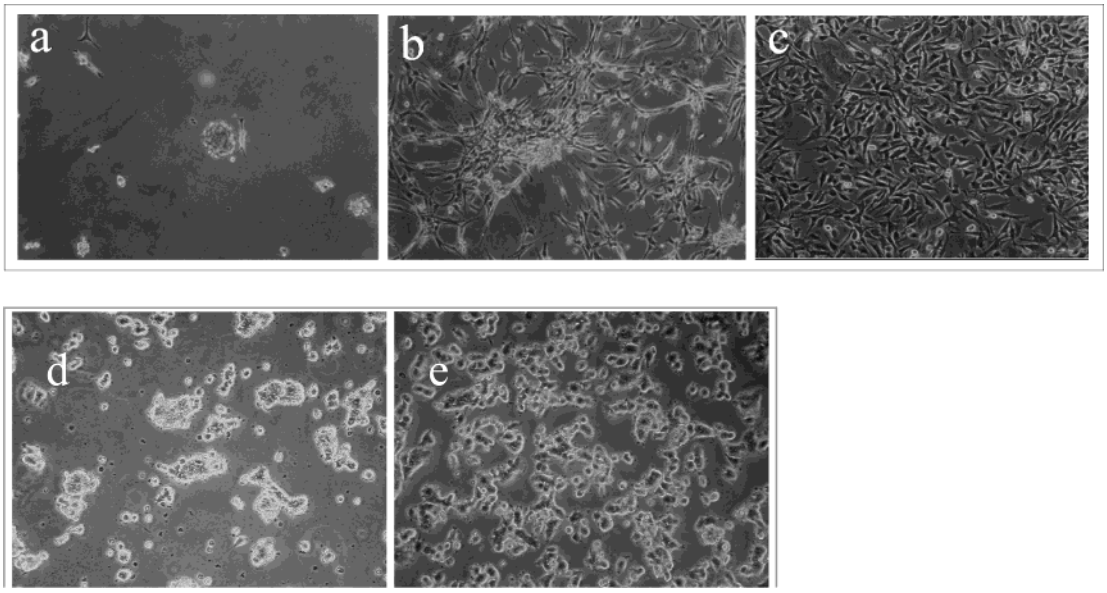

Figure 5. Optical microscopy images of C2Cl2 myoblast (a−c) and PC12 (d−e) culture cells on the surface of (a) $(PDDA/CdTe)_3$, (b) $(PDDA/CdTe)_3PDDA(PAA/collagen)_1$, (c) $(PDDA/CdTe)_3PDDA(PAA/collagen)_5$, (d) $(PDDA/CdTe)_3PDDA(PAA/collagen)_5$ (e) glass.

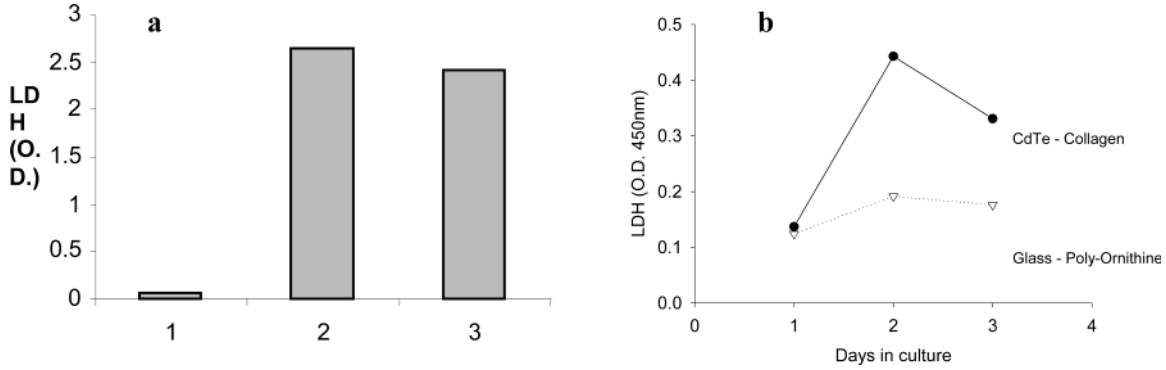

Figure 6. (a) LDH activity in media from C2Cl2 cells (1) assay medium RPMI 1640 with 0.1% fetal bovine serum; (2) $(PDDA/CdTe)_3PDDA(PAA/collagen)_5$ LBL film, and (3) control polystyrene dish; (b) LDH activity in media from PC12 cells at 1−3 d. after plating.

adsorption in the 500−600 nm region of the visible spectrum (Figure 2, trace 2), which allows the utilization of light sources that are the least damaging/dangerous to cells. If necessary, both adsorption and emission of the NPs can be further shifted to the 800−1500 nm IR region by using dopants such as Hg^{2+}.[20,21] The luminescence of the films was not affected by seeding both C2C12 and PC12 cells (see below). Visual observation indicated that it remained virtually unchanged during the culture time (several days) fading, however, after extensive exposure to open day light.

CdTe and other Cd-containing materials are very cytotoxic as evidenced by numerous reports.[22] The cytotoxicity persists in different forms of heavy metals especially in the most mobile form such as Cd^{2+}. Coating of NPs by biocompatible materials such as SiO_2,[3−5] polymers,[2] bovine serum albumin[23] or other proteins can substantially reduce the toxic effects; however, it would be important to find an approach to render NP material biocompatible, keeping the NP cores in a close proximity to facilitate signal transduction between the semiconductor material and living cells.[24]

The chemical composition of the surface makes a substantial (although not entire) contribution to biocompatibility of the material. An alternative approach to wrapping each individual NP into an insulating sheath is to coat the NP assembly as a whole by a suitable biological compound that is not toxic to living cells. This can be accomplished by LBL assembly, where a new series of bilayers can be built on top of the CdTe film, forming a "buffer zone" between the semiconductor material and effectively screening the cytotoxicity of the CdTe NPs. Many natural polymers and their analogues can render biocompatiblity to surfaces,[26,27] but probably the most widely used and best characterized cell adhesion promoter is collagen,[25,28] which was utilized in this work.

To find the most appropriate conditions for film deposition, the collagen LBL films were initially assembled separately from NPs and then integrated with the $(PDDA/CdTe)_n$ multilayers by depositing an additional PDDA layer over them. Collagen was assembled with negatively charged poly(acrylic acid), PAA, as a partner polyelectrolyte from aqueous solution at pH 4 where the protein is positively charged. PAA/TiO_2 nanoshell multilayers exhibited good biocompatibility with neuron precursor PC12 cells,[14] which also advocated the use of PAA for CdTe coating.

The structure of the $(PAA/collagen)_1$ bilayer was analyzed by AFM. In Figure 3, one can clearly see the fibrous filaments of collagen and PAA macromolecules coating the substrate uniformly and homogeneously. Importantly, the collagen layer reveals substantial porosity with diameter of channels between 10 and 50 nm. This is sufficient for the two-way traffic of many important biological molecules between the layer of NPs and cell culture, while minimizing direct contact of cells with the NP material. Figure 4 shows the dependence of the film thickness vs the number of LBL cycles for $(PAA/collagen)_n$ multilayers using elipsometry. The thickness increment initially increases (first three deposition cycles) and then becomes virtually constant. The average thickness per one (PAA/collagen) bilayer is 30 nm.

Biocompatibility is a multifactorial characteristic of the material. While correlations with chemical composition, surface charge, roughness, and porosity of the surface exist, they are not deterministic. Therefore, the actual cell survival and growth was assessed as a cumulative characteristic of biocompatibility and cell tolerance toward the stratified NP material. For that, we used mammalian C2C12 and PC12 cells in culture, which represent convenient models for expected interactions between the prepared nanoparticle/polyelectrolyte/collagen composite and tissues. When cells were cultured on $(PDDA/CdTe)_n$ multilayers, only clusters of dead cells were observed on the film surface (Figure 5a). When the CdTe NPs films were coated by a single PAA/collagen bilayer, the behavior of cells dramatically changed (Figure 5b). They attached in large quantities and spread over the surface, indicating that the cytotoxicity of the CdTe was markedly screened. They also show the signs of aggregating in large colonies. When the number of collagen bilayers was increased to $(PAA/collagen)_5$, so did the total number of cells attached: they covered the entire surface of the film in a confluent monolayer (Figure 5c). PC12 cells reacted differently. There was no survival in native (PDDA/CdTe) films (not shown), and although the addition of collagen allowed cell survival, collagen as an attachment factor was not optimal for PC12.[29] The cells tended to clump and remained loosely attached (Figure 5d) as compared to PC12 cells grown on glass (Figure 5e) treated with the standard attachment factor polyornithine. These results indicate that biocompatibility factors must be engineered to the cell type, as cell surface molecules on various cell types will determine attachment, survival, and other functional interactions between the cell and the substrate.

These results are confirmed by LDH assay. RPMI 1640 media with 0.1% FBS was used as background. The commercial polystyrene dishes were used as control. The $(PDDA/CdTe)_3$ and $(PDDA/CdTe)_3PDDA(PAA/collagen)_5$ LBL film samples prepared on polystyrene dishes were tested. Similarly to the cell adherence test (Figure 5), only stratified CdTe LBL films of with collagen on top demonstrated viable cells.

For C2C12 cells cultured in media on a $(PDDA/CdTe)_3PDDA(PAA/collagen)_5$ film, the cytotoxicity of CdTe NPs films coated by the five PAA/collagen bilayer (Figure 6a) was as same as a control polystyrene dish, which is quite remarkable and correlates well with the cell adherence in Figure 5c. As expected, somewhat higher levels of LDH were observed for PC12 cells cultured on $(PDDA/CdTe)_3PDDA(PAA/collagen)_5$. Additionally, we tested the dynamics of LDH concentration over 3 days. The stratified NP films were placed in six-well plates, cells were seeded, and the media that were collected remained unchanged over the entire experiment. At day 1, PC12 grown on $(PDDA/CdTe)_3PDDA(PAA/collagen)_5$ showed identical survival to PC12 grown on plain tissue-culture treated glass. On day 2, PC12 cells showed high LDH values because although they were alive, the collagen substrate did not support firm attachment (Figure 6b; see also Figure 5d). Interestingly on day 3, the level of LDH dropped noticeably, indicating probably stronger cell adhesion.

The tremendous improvement of biocompatibility of the NP films by adding the layer of collagen on top of it should be attributed to the elimination of direct contact of the cells with the semiconductor surface. However, we also found that the elution of soluble forms of Cd^{2+} in the media is also greatly reduced in the stratified layers. The equal amount of medium was exposed $(PDDA/CdTe)_3PDDA$ and $(PDDA/CdTe)_3PDDA(PAA/collagen)_5$ for 5 days. The concentration of Cd^{2+} in the media collected over $(PDDA/CdTe)_3$ films was 0.080 mg/L, while that in the media from the $(PDDA/CdTe)_3PDDA(PAA/collagen)_5$ sample was only 0.0025 mg/L; i.e., 32-fold decrease as compared to $(PDDA/CdTe)_3$. Therefore, the improvement in biological properties of the multilayers partially comes from deceleration of NP decomposition in the film after coating with collagen.

Conclusion. Improved biocompatibility of CdTe/PDDA multilayers is the first step toward the study of interactions between NP/polymer composites and living tissues. Although Cd-containing materials are unlikely candidates for in vivo use, ex vivo models with these particles can provide interesting information on fundamental problems at the interface of nanomaterials and biomaterials. Additionally, the described LBL procedure can also be considered as a general approach to rendering surfaces biocompatible. As the data presented here demonstrate, even highly cytotoxic surfaces can be modified to alleviate/eliminate this problem. At the same time, cell viability may not be the only necessary requirements for an interface between a man made and living system. Surface of nanostructured composites will need to be engineered to confer not only cell survival but also functionality of both the man made and the biological materials at the interface. We believe that the universality and flexibility of the LBL design makes functional adaptation very feasible and makes LBL one of the most potent approaches for the materials design in the biomedical field.

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