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Free-Standing Cell Sheet Assembled with Ultrathin Extracellular Matrix as an Innovative Approach for Biomimetic Tissues

*Jun Chen, Xiaozhong Qiu, Leyu Wang, Wen Zhong, Jiming Kong, and Malcolm M. Q. Xing**

Current artificial tissue-substitutes have limited clinical applications due to unmatched complex combination of cells and extracellular matrix (ECM) as seen in native tissues. From a developmental perspective, the construction of effective biomimetic tissues is from the bottom (one-dimensional nanoparticles or two-dimensional membranes) up (three-dimensional scaffolds or more complex composite). In a hierarchical architecture, each sub-structure can be assembled in a flexible way with specific regulators and cells, which overcomes the deficiency of one-for-all scaffold. Here, a cell-compatible cell-lined layered nano-membrane is developed. Bioactive molecules are mounted on a nano-membrane and later released to its lined cell sheet. The cell-lined membrane is in a free-standing form to regulate cellular functions. The major advantage of this methodology is to provide a versatile approach to construct biomimetic tissues for clinical applications.

1. Introduction

In the last decade, lab-research achievements have been made in the fabricating and engineering of soft tissues. Significant efforts have been made to fabricate numerous biomedical materials for this purpose including hydrogels, porous materials and nanoparticles aggregations.[1–3] A key shortcoming existed in the initial premise of the traditional approach towards scaffold design, which was to prepare a substrate to support cell attachment and growth.[3] The resulted disadvantages, for example, the poor ratio of functional cells to synthetic materials,[4,5] their isotropic nature,[5] the single function and non-uniform cell penetration,[6] and so on, compromise the effect of as-built artificial tissues. A rational approach is urgently needed to fabricate multifunctional materials to match the complicated biomedical uses via architecting materials with hierarchic and organized structures. Of several available strategies, layered cellular assembly approaches were developed to improve controlling over tissue organization.[7,8] As one of two kinds of cellular assembly approaches, the strategy of assembling cell sheets to fabricate stratified tissues was first explored by Okano and co-workers,[9] who employed a temperature-sensitive coating on cell culture substrates that undergoes hydrophobicity-hydrophilicity change under change of temperature.[10] Confluent cell sheets could thereby be retrieved by simply cooling the substrate without adding enzyme. The successively sophisticated studies on tissues assembling from stacking cell sheets demonstrated a successful fashion to build up the complex tissues.[7,11–13] Despite many advantages of cell-sheet approaches, several challenges still impede its progress, for example, the lack of controlling the cell fate and poor mechanical stability of cell sheet.[14]

To meet the above requirements, use of a free-standing cell sheet with layer-by-layer (LbL) ultrathin films as "nano-clothing" on it may provide us a pragmatic approach. First, the synthetic polymers have much higher mechanical strength than pure cell sheets at the cost of minimal amount of polymeric scaffold to

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achieve effective structural reinforcement. Consequently the cell sheet is easier to be handled for favoring the stacking construction of tissues. Another significant advantage in the use of LbL ultrathin films to build tissue scaffold comes from the bottom-up approach.[15] The mechanism of molecular self-assembling on the cell surfaces drives the as-built structural support with a maximum homogenized mechanical effect to the cells, highly customized for maintaining cell prototypes. The free-standing planar films are highly amenable to surface modification and engineering to match the various ends in specific tissue compartment. And the controlled release of desirable drugs or growth factors can be achieved by simply encapsulating cargos into the ultrathin films to affect the cell fate in the cell sheet.

In this perspective, we report a cell-compatible free-standing cell-lined layered nano-membrane. Bioactive molecules were mounted on a nano-membrane and later released to its lined cell sheet (**Figure 1**). The cell-lined membrane can be a free-standing form to regulate cellular functions, and also can be assembled with different types of cells. The major advantage of this approach is to provide an alternative and flexible approach to construct biomimetic tissues for the application in translational medicine.

2. Result and Discussion

The developments of cell-polymeric composite materials have resulted in remarkable advances in tissue engineering. The drawbacks existing in the prevalent strategies make it an urgent demand to develop an effective methodology of fabricating the free-standing multilayered films composed of desired cells and nanometer scaled extracellular matrix. In this study, the process of obtaining the free-standing nanomembrane lined with cells was performed according to the Figure 1. First, we constructed the cell detachable surfaces following the well-established method devised by Okano and colleagues with minor modifications.[9,12,13,16,17] Poly (N-isopropylacrylamide) (PNIPAM) is a temperature-responsive polymer with a LCST of 32 °C.[17–19] We grafted temperature-responsive PNIPAM onto glass slides by a

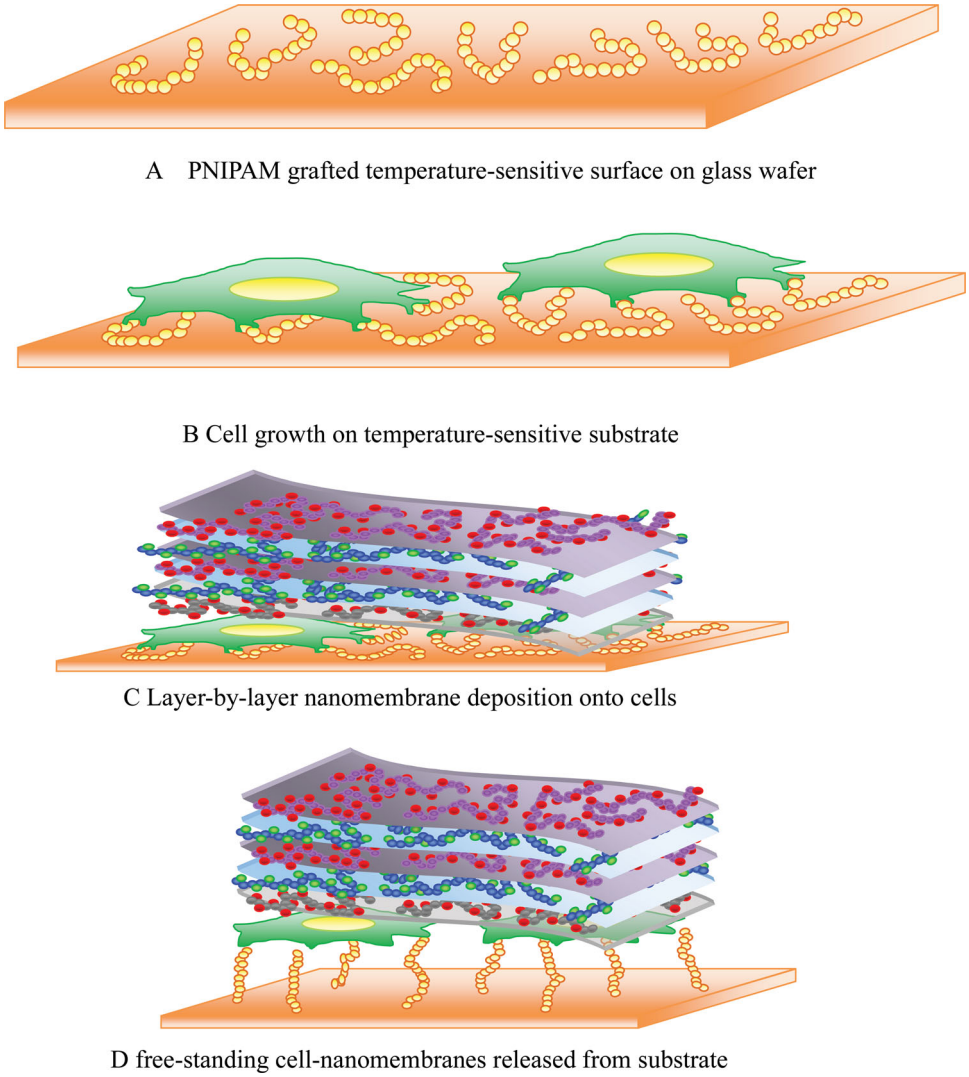


Figure 1. Schematic illustration of fabrication of free-standing cell-nanomembranes.

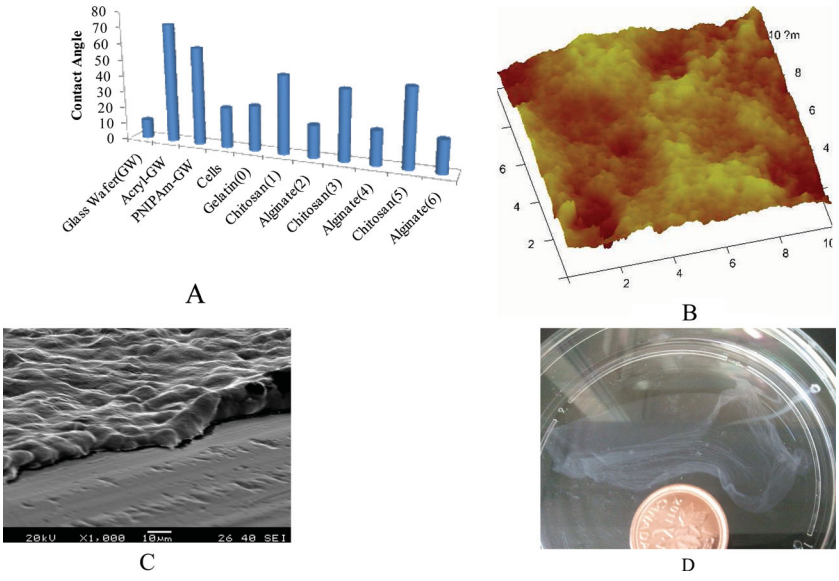


Figure 2. A) The contact angles of various surfaces in the process of preparation of the free-standing film. B,C) AFM and SEM (scanning electron microscopy) images of the morphology of a free-standing nanomembrane-cell sheet. D) Photo of free-standing film floating in a PBS buffer.

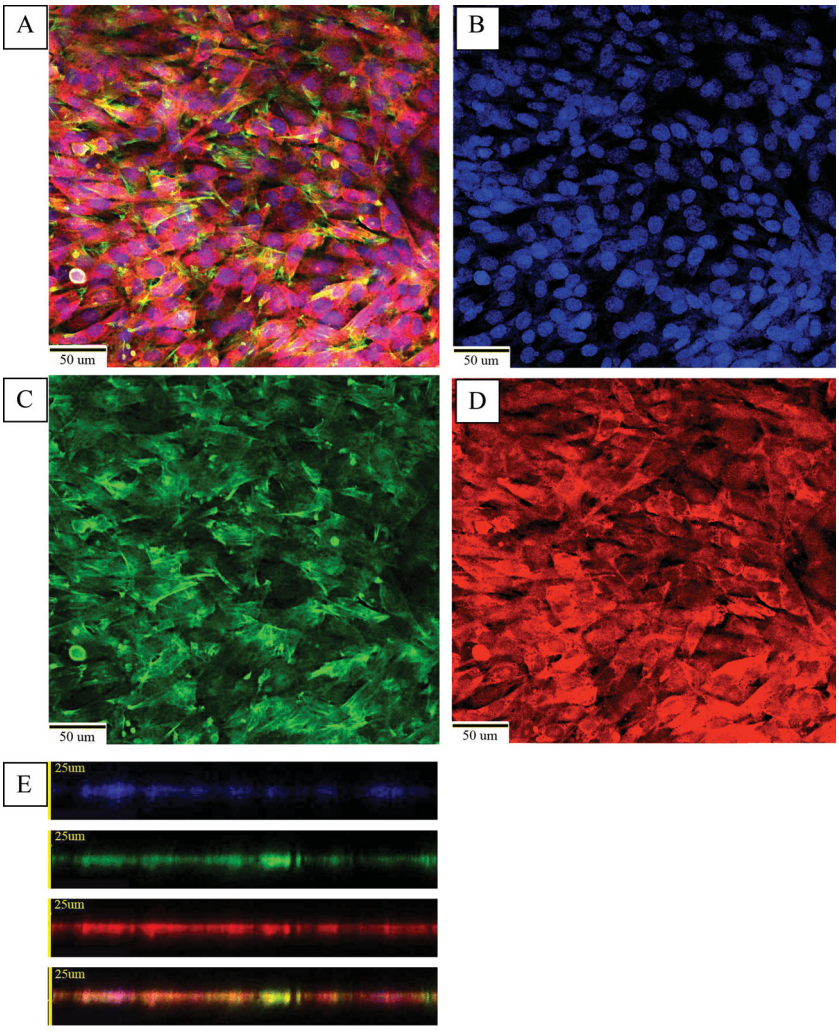
two-step method depicted in Figure S1 (Supporting Information): A self-assembled silane monolayer was formed by hydrolyzing 3-acryloxypropyltrimethoxysilane (APTES) on a clean glass surface.[20] Then, the graft polymerization of NIPAM was carried out.[21] The contact angles of the glass substrates were monitored during surface treatments. As shown in **Figure 2**A, the contact angles changed after each treatment. The contact angle was 12° on the whole surface, indicating that the substrate had been uniformly covered with hydroxyl groups after piranha solution.[8,18] Further, the hydroxylated surface was coated with APTES for 24 h via the hydrolysis of the silane groups, leading to a rise in the contact angle to 72°. When a PNIPAM layer was formed on the surface, the contact angle decreased to 60°. Atomic force microscopy (AFM) was also employed to evaluate the surface morphology of the two substrates: APTES modified and PNIPAM-modified surfaces. Figures 2A and B showed thedeposited silane layer and PNIPAM-grafted surface. Figures 2C shows typical IR absorbance bands at 1644.2 and 1539.9 for amide bond stretching and the band at 2971.7 for CH_3 stretching and at 3284.3 for N–H stretching, which presents PNIPAM grafted on the substrate.

LbL deposition is a well-studied method of preparing nano-scaled films on a substrate.[22–25] But only a few reports described using this method directed onto the surface of cells. The cells were firstly cultured on the hydrophobic PNIPAM layer on the petri-dishes. Once 80–90% confluence of the cells was achieved, the layer-by-layer process was conducted on the surface of the cell sheet. A key issue is to keep cell viability during LbL deposition process. Inspired by the study of Akashi et al.,[26] which reported the preparation of a multilayered cell structure combined with a multilayer membrane composed of component gelatin as extracellular matrix.[1,5,27] We employed gelatin as the cell-contacting layer and chitosan-alginate as the nano-matrix.[13,28] Gelatin is a natural biocompatible polyelectrolyte (PE) providing excess charges. Gelatin layer is considered a key factor for layer-by-layer self-assembly[29] on the surface of cell sheet to keep high cell viability during PE depositing on cell sheet. Alternate-charged PE layers of chitosan and alginate with 0.1 w/v% were assembled onto the gelatin-coated human myoblast cells C2C12 or mesenchymal stem cells on the PNIPAM-grafted surfaces as sketched in Figure 1. The contact angles of the surfaces after different steps were measured to evaluate the layer-by-layer deposition of the various polyelectrolytes. As shown in Figure 2A, the contact angle of the cell surface was about 22°, and the contact angle increased to 25° after the application of a gelatin coating. The deposition of a chitosan layer on the gelatin increased the contact angle to about 45°. The following deposited alginate layer reduced the contact angle to around 20°. The final contact angle of the assembly was about 20°. The contact angle results demonstrated the successful deposition onto cells through layer-by-layer fashion. The fabrication of multilayer membranes composed of ECM-related components on the surface of the cells provides a cell-adhesive surface to efficiently interact with the cells.[26] The charges on these components can facilitate the subsequent deposition of another layer of polymelectrolyte.[22,24]

The morphology of a free-standing cell-nanomembrane is shown in Figure 2B,C. In order to investigate the morphology of cell nanomembranes, gelatin–(rhodamine-chitosan/alginate)$_3$ membranes were prepared on the model cells, human myoblast C_2C_{12}. CLSM images of the top view and cross sections of the nanomembranes show a homogeneous assembly of gelatin–(chitosan/alginate)$_3$ nanofilms on the surface of the cell sheet (**Figure 3**E). The obtained homogeneous assembly was in accordance to many reports of layer-by-layer research, which indicated that the moving ability of soft polymer chains drives them "in and out" of the films during the immersion process and finally forms the entangling and diffusion of the polyelectrolytes (in particular weak polyelectrolytes) in the whole as-built multilayered films.[22,24,25]

To evaluate the compatibility of the nanomembranes on cells, the live/dead assay of the gelatin–(chitosan/alginate)$_3$ nanofilms on the cell surface was conducted. The result shows 99% cell viability, revealing the important role of gelatin as the first layer next to the cells (Figure 3F). The immunostaining images also present a monolayer of healthy cells (Figure 3A–D).

Despite extensive studies have been carried out on the multilayered ultrathin film, the preparation of the free-standing ultrathin multilayered films is still a great challenge for the researchers. The major obstacle existed in the strong adhesive force between the films and planar substrates and the weak mechanical strength of the as-prepared multilayered assemblies. As such, several strategies have been developed in the effort to overcome this conflict. One method is proposed to weaken the interaction between the multilayered films and the substrates. Thereby either a dissociable layer between the substrate and multilayered films or the removable substrate was applied as a sacrifice layer. For example, Dr. Ono and Dr. Decher depicted a multilayered film (PAH-PSS) on top of a hydrogen-bond based



(Blue color is attributed to the nuclei stained by Topro-3 and transfer to blue color using software; green color attributed to the F-actin in the cytoskeleton stained by phallacidin; and red color attributed to the rhodamine labeled chitosan (Rh-Chitosan)).

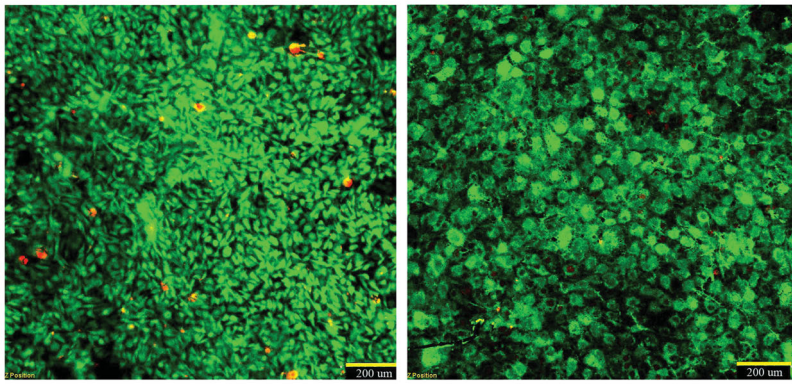


Figure 3. A–D) CLSM images of C2C12 cell-gelatin-(rh-chitosan/alginate)$_3$ nanomembranes and E) cross-section scanning images of the free-standing cell nanombranes. F) Live/Dead assay of gelatin-(chitosan/alginate)$_3$ deposition on the C2C12 cell surface. G) Live/Dead image of free-standing C2C12 cell-gelatin- (chitosan/alginate)$_3$ nanomembranes detached from the thermo responsive surface. The scale bar is 200 um.

film (PAA-PEO) in acidic medium. When subsequent increasing of pH value to 7, the sacrificial PAA-PEO layers dissolved to release the electrostatically bonded PAH-PSS multilayered films.[30] The other approaches have been reported to liberate the multilayered film by enhancing the mechanical strength of multilayered assemblies. These methods involve increasing the bilayers number, incorporation of inorganic composites and chemical crosslinking. For example, Takeoka group developed the method to prepare the multilayered films on the hydrophobic surface, and with the increasing of layers number, the multilayered films became robust enough to be peeled off.[31] In some cases, a more robust sacrifice layer, such as PVA layer was cast on top of the multilayered film to rapidly increase the mechanical strength.

However, there are distinct flaws in those proposed strategies: time-consuming, harsh chemical reactions, the compromise of the biocompatibility of as-prepared materials. In this study, the transition of surface wettability at a lowered temperature was exploited to allow cell sheet to detach spontaneously from the grafted surfaces. Briefly, the free-standing cell–gelatin–(chitosan/alginate)$_3$ films was obtained by placing the dish in a cold room (4 °C) for 1 h or room temperature for 2–3 h. The cell–gelatin–(chitosan/alginate)$_3$ membrane was first rolled up from the edge and then the whole film was detached from the substrate with a PNIPAM-grafted surfaces with the minor use of cell lifter. The liberation process of the multilayered film was mild and physiological. With the assistance of the cell sheet, this multilayer assembly was robust to be able to be manipulated in the aqueous solution or even in air. Such a simple, mild, facile approach may provide us a novel methodology towards the preparation of free-standing ultrathin films. Similarly, the live/dead assay of the free-standing cell-gelatin–(chitosan/alginate)$_3$ multilayered films was conducted. The lined cells maintained 90% viability after the detachment procedure (Figure 3G). The results suggest that the developed methods are cell-compatible.

As discussed above, one of the key advantages of the design in this study is to endow the free-standing stratified hybrid membrane with the ability of regulating cell functions in-situ to favor tissue engineering applications. BMSCs have the capacity to differentiate into a variety of cell types and have been widely used in tissue engineering.[32,33] The differentiation of MSCs can be governed by chemical and physical cues including

micro- and nano-topography, and even rigidity of the substrate, as the differentiation of MSCs usually is in vivo when MSCs are embedded in the ECMS.[34,35] Bone morphogenetic proteins (BMPs) play a key role in osteogenic differentiation and bone development and can drive uncommitted mesenchymal precursor cells toward the osteoblast lineage.[36,37] The concentration of BMP2 usually used for inducing MSCs or preosteoblasts differentiation in vitro is 50–200 ng mL^{-1}. During the inducing process, some other inducer (including dexamethasone, ascorbic acid, or β-glycerol phosphate, etc.) were added into the basic media to help the inducing effect of BMP2.[36]

To investigate the effects of regulator-loaded membranes on the osteogenic differentiation of stem cells, a free-standing membrane composed of BMP2-loaded gelatin-(chitosan/alginate)$_3$ membranes lined with mouse BMSCs was fabricated utilizing electrostatic interactions between the protein and polymer to mediate assembly and characterized. In this study, we used a low concentration of BMP2 (10 ng mL^{-1}) and basic media to investigate the effect of gelatin-(chitosan/alginate)$_3$ film on the osteogenic differentiation of BMSCs. Prior to this investigation, the in-vitro release profiles of a model protein FITC-BSA from PEI/alginate-gelatin-(chitosan/alginate)$_3$ and PEI/alginate-gelatin-(chitosan/alginate)$_6$ on the substrate was firstly investigated by monitoring fluorescein conjugated BSA. We prepared PEM films on glass slides utilizing electrostatic interactions between the protein and polymer to mediate assembly as the method introduced above. As shown in Figure S3 (Supporting Information), the release of protein exhibited a slow and long-period protein-release mode for the two samples. A slight increase of protein release and similar release behavior from PEI/alginate-gelatin-(chitosan/alginate)6 was found in the 7-day release course as compared to the PEI/alginate-gelatin-(chitosan/alginate)3. The superior feature of polyelectrolytes layer-by-layer multilayered films is to provide great promise for the release of laden cargos in a controllable manner. Diverse multilayered films have been investigated for the controlled release of encapsulated agents relying on the disassembly of the films.[24,38,39] In this study, the disassembly of the multilayered films was dominated mostly by the slow degradation of chitosan and alginate, which led to the slow and long-period protein release behavior. In addition, the strong hydration nature of the component polymers in the films, like chitosan and alginate, could be potentially advantageous for loosing association of the films by extending the distance between charges thus weakening the electrostatic interaction strength.[38] This effect is believed to be a cumulative process with the layer number increasing, which may result in the slight release increase of protein from PEI/alginate-gelatin-(chitosan/alginate)6.

We compared the gene expressions of bone sialoprotein (BSP), runt-related transcription factor 2(Runx2), collagen type I(COL-I), osteopontin(OPN) in cells grown on tissue culture polystyrene (TCPS), TCPS + BMP2 and free-standing cell-gelatin-(chitosan/alginate)$_3$-BMP2 (film). These proteins are considered lineage-specific markers of osteoblastic differentiation.[32,34,40] Runx2 is essential for osteoblastic differentiation and bone formation. COL-I is the major organic component of bone matrix produced by osteoblasts. It functions as a scaffold of mineralization in bone. Bone sialoprotein (BSP) is a highly sulfated, phosphorylated, and glycosylated protein that mediates cell attachment. Osteopontin is a phosphoprotein member of the SIBLING family that possesses several calcium-binding domains and is associated with cell attachment, proliferation, and mineralization of extracellular matrix into bone, synthesized by bone-forming cells. As summarized in **Figure 4**, in quantitative real-time RT-PCR (**qPCR)**, mRNA expression of Runx2, COL-I, and OPN in the TCPS + BMP2 group on day 7 decreased significantly as compared to the TCPS + BMP2 group on day 3. Whereas, on day 7, the expression levels of four genes in the cell-gelatin-(chitosan/ alginate)$_3$-BMP2 group were significantly increased than those in the cell-gelatin-(chitosan/alginate)$_3$-BMP2 group on day 3. Compared to the TCPS + BMP2 and TCPs groups on day 7, Runx2, COL-I, BSP, and OPN mRNA expression in the cell-gelatin-(chitosan/alginate)$_3$-BMP2 group elicited respectively an ≈14-fold, ≈13-fold, ≈3.7-fold, and ≈19-fold increase as compared to the group of TCPS + BMP2, and elicited a ≈1.5-fold, ≈1.3-fold, ≈30-fold, and ≈1.9-fold increase, respectively, as compared to the TCPS group. In the present study, the expression of four osteoblastic-specific genes in BMSCs in the group of TCPS + BMP2 all decreased except BSP. This suggests that the low concentration and one-time administration of BMP2 protein alone provides less induction of osteogenic differentiation of BMSCs particularly for a long time scale, as the osteogenic effect of BMP2 has a close relationship with its concentration. Whilst the expression of these four genes in BMSCs in the group of gelatin-(chitosan/alginate)$_3$-BMP2 exhibited significant increase on day 7 as compared to those on day 3, and the TCPS and TCPS + BMP2 groups on day 7. Combined the in-vitro release profile of the multilayered films, it can be explained that sustainable release of the loaded BMP2 from the multilayered films assisted the induction of osteogenic differentiation of BMSCs in a long time scale. At initial stage, the release amount of BMP2 from the multilayered films was probably too less to regulate the induction of osteogenic differentiation of BMSCs especially in contrast to the one-time administration of 10 ng mL^{-1} BMP2. Nevertheless, the sustainable release behavior of inappreciable dose of BMP2 from the multilayered films exhibited the surprising appreciable ability of inducing the osteogenesis of BMSCs in a long term. In addition, our proposed cell sheets were completely upheld on the multilayered films instead of the traditional cell culture dishes. This long-term ability of enhancing the osteogenic differentiation of mouse BMSCs and the free-standing soft property make it very suitable for the potential application of tissue regeneration.

In summary, a novel strategy to fabricate a free-standing multilayer nanomembrane lined with a cell sheet was developed in the present work. The complex film could be peeled from the substrate upon temperature changes to form a free-standing planar cell-nanomembrane. The architecture was well organized, even after the complex was peeled off the substrate. The scaffold mechanically supported the cell sheets and maintained the cell proliferation at the minimal cost of adding extra polymer components on the cells. This feature may point out the way out of the dilemma existing in the traditional scaffold, minimizing the ratio of the functional cells to the synthetic materials to obtain the greater overall functions. The gelatin–(chitosan/alginate)$_3$ assemblies exhibited good cell compatibility to meet the fundamental requirement for the

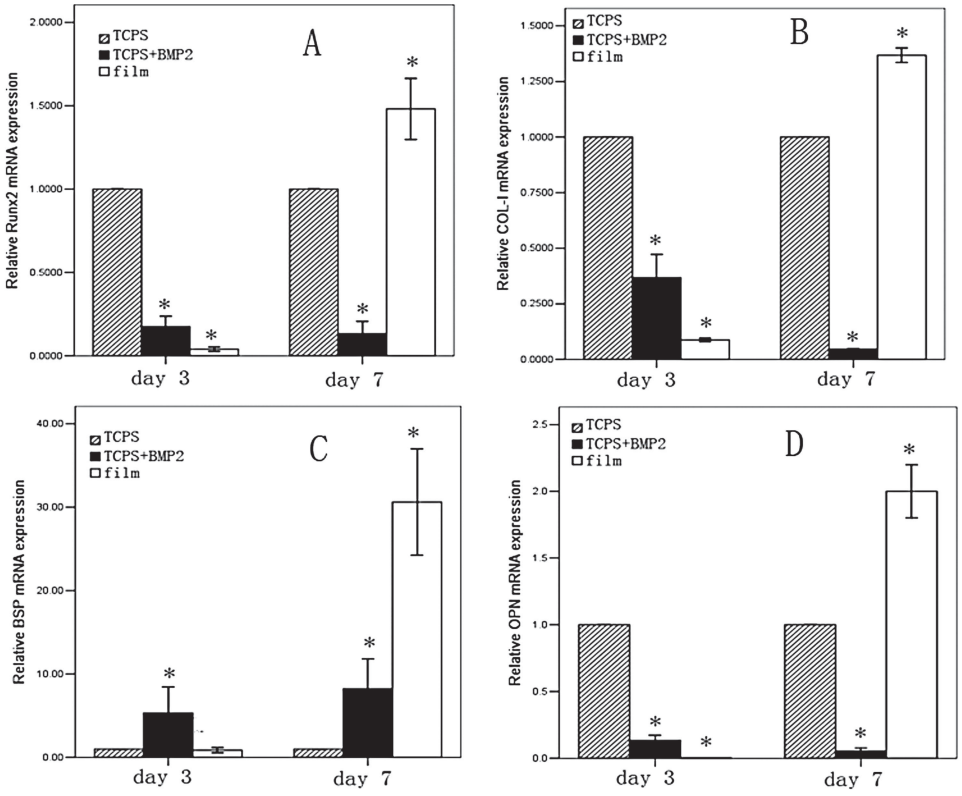


Figure 4. Real time-qPCR of osteogenic gene expression levels of mouse BMSCs cultured in vitro. Total RNA was prepared from BMSCs grown on either TCPS, TCPS + BMP2 or gelatin-(chitosan/alginate)3-BMP2 film for three or seven days. A) Runx2, B) COL-I , C) BSP, and D) OPN gene expression was quantified using real time-qPCR methods; GAPDH was used as an internal control. Data values are expressed as mean ± SE (n = 3). "*" means $p < 0.05$ vs the TCPS group.

consecutive application of the tissue construction. Most significantly, the ability of nanomembrane to control the release of loading drugs and growth factors provided us a potent approach to control the cell differentiation in the scaffold. To the best of our knowledge, this is the first effort to fabricate free-standing, cell-lined, and multilayered membrane via layer-by-layer deposition as an ECM to regulate cell differentiation. The planar free-standing nanomembranes can be potentially used as "bricks" and "concrete" at same time to fabricate various layered complex assembly with target cells and regulators to prepare the complex soft tissues.

3. Experimental Section

Materials: The glass slides, N-isopropylacrylamide (NIPAM) and 3-acryloxypropyl- trimethoxysilane (APTES) were purchased from Aldrich Chemical Co. All solvents were purchased from VWR Canada Chemical Co. and used as received.

Glass Substrate Pretreatment: The glass slides were first rinsed with a large amount of chloroform, then ethanol and finally distilled water. After that, the slides were immersed for one hour in a piranha solution, which is composed of a mixture of 70 v/v% concentrated sulfuric acid (98 wt%) and 30% volume of a hydrogen peroxide solution (30 wt%). The cleaned glass wafers were washed with a large amount of distilled water and ethanol, and then dried in a vacuum oven for 24 h. The contact angle of water droplets on the pretreated glass surface was about 15 ° over the entire surface.

Silane Modification on the Glass Surface: Hydroxylated glass wafers were placed into a 3 wt% toluene solution of APTES and refluxed under nitrogen atmosphere for 24 h. After the hydrolysis, the glass slides were ultra-sonicated in toluene for 5 min. Then they were rinsed with acetone and ethanol and then dried in a vacuum.

Surface-Initiated Graft Polymerization of NIPAM: For the surface-initiated graft polymerization with NIPAM, the slides were immersed in a solution of 2% (v/v) photo-initiator (1,1′-Azobis (cyclohexanecarbonitrile)). NIPAM was spin-coated onto the 3-acryloxypropyltrimethoxysilane-modified glass wafers at 800 rpm for 30 s. The slides were irradiated through a photomask for 5 min by a 365 nm-UV light source (80mW/cm^2). After the reaction, the PNIPAM-grafted glass wafers were rinsed thoroughly under ultrasonic bath of ethanol and distilled water and then dried in a vacuum.

Characterization of the Surface Modified Glass Wafers: The graft modified glass surface was characterized by contact angle, ATR and AFM. The water contact angles of the slide and thereafter the coatings of cells and nanomembrane were measured in the ambient air using a CA-A contact angle measuring system. Each value reported in this work is the averaged value of four measurements on different locations of the same surface. Tapping-mode AFM analysis was carried out using the microscope system. The image was acquired by using standard silicon TESP probes (their nominal spring constant and resonance frequency are 50 N m^{-1} and 300 kHz, respectively).

Cell Culture: Human C2C12 cells (gifted from Dr. K Wrogemann, University of Manitoba) were seeded onto the PNIPAM-grafted slides and cultured with a Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 units mL^{-1} penicillin. Mouse bone marrow mesenchymal Stem Cells (BMSCs) were purchased from the American Type Culture Collection (ATCC, USA).

Table 1. Sequences of primers for the qRT-PCR.

Gene	Forward primer sequence	Reverse primer sequence
BSP	5′-ccacactttccacactctcg-3′	5′-cgtcgctttccttcacttttg-3′
Runx2	5′-gctattaaagtgacagtggacgg -3′	5′-ggcgatcagagaacaaactagg -3′
COL-I	5′-aacagtcgcttcacctacag -3′	5′-aatgtccaagggagccac -3′
OPN	5′-ctacgaccatgagattggcag-3′	5′1-catgtggctataggatctggg -3′
GAPDH	5′-aggtcggtgtgaacggatttg-3′	5′-tgtagaccatgtagttgaggtca-3′

BSP bone sialoprotein, Runx2 Runt-related transcription factor 2, COL-I collagen type I, OPN osteopontin, GAPDH glyceraldehyde-3-phosphate dehydrogenase.

Mouse BMSCs were maintained in DMEM with high glucose. The media were supplemented with 10% FBS and 1% penicillin/streptomycin. All cells were incubated at 37 °C in a humidified incubator containing 5% CO_2. The medium was changed every three days.

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR): To analyze osteogenic differentiation of BMSCs, the cells were divided into three groups: aTCPS group, a TCPS + BMP2 (10 ng/ml, ProSpec, USA) group, and a cell-gelatin-(chitosan/alginate)$_3$-BMP2 group. In the cell-gelatin- (chitosan / alginate)$_3$ -BMP2 group, the cell density was 2×10^6 cells mL^{-1}.

RNA was extracted from cell layers at days 3 and 7 using TRIZOL reagent (Invitrogen). Starting from 1 μg RNA, 20 μL cDNA were synthesized using Verso cDNA kit (GeneCopoeia Inc, USA) with oligo-dT primer in the presence of dNTP. Then quantitative real-time PCR was performed by SYBER Green assays (GeneCopoeia Inc, USA). Amplification conditions were as follows: Hold in 95 °C for 10 min, followed with 40 cycles at 15 s in 95 °C and 1 min in 60 °C. Thermal cycling and fluorescence detection were done using the StepOnePlus Real-Time PCR System (Applied biosystems, USA). The mRNA expression levels were determined relative to the GAPDH by the ΔCt method. Primer sequences are shown in **Table 1**.

Preparation and Characterization of LBL Assembly of Gelatin-(chitosan-alginate)$_3$ on a Monolayer of Cells: A type A Gelatin (Sigma-Aldrich) solution was prepared by dissolving the polymer in PBS at pH 7.4 at a concentration of 0.5% (w/v). A chitosan (medium molecular weight, Sigma-Aldrich) solution (concentration: 0.1% w/v) was prepared by dissolving the polymer in 1% (v/v) acetic acid; this solution maintained a pH between 6.1 and 6.3. Alginic acid (Sigma-Aldrich) was diluted in PBS at a concentration of 0.1% (w/v) and maintained at the physiological pH (7.4). The samples were rinsed with 1× PBS (warmed to 37 °C) prior to PE deposition. All the solutions were pre-warmed to 37 °C before being applied to the LbL deposition. First, a gelatin layer was deposited on the cell layer by carefully adding 1 mL of gelatin solution using a pipette and the dish being put in an incubator for 10 min. The gelatin solution was then removed by aspiration. After that, a cationic polyelectrolyte (PE) layer was deposited on top of the gelatin layer by carefully adding 1 mL pre-warmed chitosan solution. After being incubated in an incubator for 5 min, the cationic solution was removed and subsequently topped by an equal volume of the anionic PE solution. This procedure was repeated by the sequential deposition of oppositely charged PE layers. At the end of the deposition procedure, the samples were rinsed in 1 × PBS (pre-warmed to 37 °C) and subsequently maintained in appropriate cell-culture medium at a 37 °C incubator.

After the LbL assembly of gelatin-(chitosan-alginate)$_3$ on a monolayer of cells, the film was washed with cold PBS three times, and cells were fixed by 4% paraformaldehyde in PBS at room temperature for 15 min. After fixation, the cells were permeabilized by 0.1% Triton X-100 in PBS for 10 min. Then the cells were rinsed with PBS three times. The cells were incubated in 10 μM phallacidin/1% (w/v) BSA solution for 20 min followed and then rinsed with PBS three times. The cells were then incubated in 10 μM Topro-3 for 20 min and then washed with by PBS three times. The cover slip was set on a microscope slide and examined by CLSM.

Vitality of the cells that had been coated with the LbL assembly of gelatin-(chitosan-alginate)$_3$ was investigated using the LIVE/DEAD Viability/Cytotoxicity Kit (Molecular Probes, L3224). With the kit, live and dead cells can be identified on the basis of their membrane integrity and esterase activity. Briefly, after removing the culture medium, the samples were rinsed with PBS. 2 μM calcein AM and 4 μM EthD-1 in PBS were added. The samples were incubated at 37 °C for 10 min and observed using the CLSM.

Preparation and Characterization of Free-Standing LbL Assembly of Cell-Gelatin-(chitosan-alginate)$_3$: The film was immersed in a cold PBS solution and kept in 4 °C for 1 h or room temperature for 2–3 h to allow the formation of the free-standing cell–gelatin–(chitosan/ alginate)$_3$. The film was then washed with cold PBS for three times, and cells were fixed by 4% paraformaldehyde in PBS at room temperature for 15 min. After fixation, the cells were permeabilized by 0.1% Triton X-100 in PBS for 10 min; then the cells were rinsed by PBS three times. The cells were incubated in 10 μM phallacidin/ 1% (w/v) BSA solution for 20 min followed by PBS rinsing three times. The cells were then incubated in 10 μM Topro-3 for 20 min, followed by PBS wash three times. The film was set on a microscope slide and examined by CLSM.

Vitality of cells in the free-standing film was investigated using the LIVE/DEAD Viability/Cytotoxicity Kit (Molecular Probes, L3224). With this kit, live and dead cells can be identified on the basis of their membrane integrity and esterase activity. Briefly, after removing the culture medium, the samples were rinsed with PBS. 2 μM calcein AM and 4 μM EthD-1 in PBS were added. Then the samples were incubated at 37 °C for 10 min and observed using CLSM.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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[1] E. Alsberg, K. W. Anderson, A. Albeiruti, J. A. Rowley, D. J. Mooney, *Proc. Natl. Acad. Sci. U. S. A.* **2002**, *99*, 12025.
[2] a) R. Langer, J. P. Vacanti, *Science* **1993**, *260*, 920; b) S. J. Hollister, *Nat. Mater.* **2005**, *4*, 518.
[3] T. Weigel, G. Schinkel, A. Lendlein, *Expert Rev. Med. Dev.* **2006**, *3*, 835.
[4] F. Yang, R. Murugan, S. Wang, S. Ramakrishna, *Biomaterials* **2005**, *26*, 2603.
[5] J. A. Rowley, G. Madlambayan, D. J. Mooney, *Biomaterials* **1999**, *20*, 45.
[6] S. F. Yang, K. F. Leong, Z. H. Du, C. K. Chua, *Tissue Eng.* **2001**, *7*, 679.
[7] a) K. Ohashi, T. Yokoyama, M. Yamato, H. Kuge, H. Kanehiro, M. Tsutsumi, T. Amanuma, H. Iwata, J. Yang, T. Okano, Y. Nakajima,

Nat. Med. **2007**, *13*, 880; b) N. L'Heureux, S. Paquet, R. Labbe, L. Germain, F. A. Auger, *FASEB J.* **1998**, *12*, 47.
[8] M. Yamato, Y. Akiyama, J. Kobayashi, J. Yang, A. Kikuchi, T. Okano, *Prog. Polymer Sci.* **2007**, *32*, 1123.
[9] T. Okano, N. Yamada, H. Sakai, Y. Sakurai, *J. Biomed. Mater. Res.* **1993**, *27*, 1243.
[10] a) P. K. Chu, J. Y. Chen, L. P. Wang, N. Huang, *Mater. Sci. Eng. R* **2002**, *36*, 143; b) M. Yamato, C. Konno, M. Utsumi, A. Kikuchi, T. Okano, *Biomaterials* **2002**, *23*, 561.
[11] K. Nishida, M. Yamato, Y. Hayashida, K. Watanabe, K. Yamamoto, E. Adachi, S. Nagai, A. Kikuchi, N. Maeda, H. Watanabe, T. Okano, Y. Tano, *New Engl. J. Med.* **2004**, *351*, 1187.
[12] T. Shimizu, M. Yamato, Y. Isoi, T. Akutsu, T. Setomaru, K. Abe, A. Kikuchi, M. Umezu, T. Okano, *Circulation Res.* **2002**, *90*, E40.
[13] T. Shimizu, M. Yamato, A. Kikuchi, T. Okano, *Biomaterials* **2003**, *24*, 2309.
[14] R. M. P. Da Silva, J. F. Mano, R. L. Reis, *Trends Biotechnol.* **2007**, *25*, 577.
[15] C. C. Co, Y. C. Wang, C. C. Ho, *J. Am. Chem. Soc.* **2005**, *127*, 1598.
[16] a) A. Kushida, M. Yamato, C. Konno, A. Kikuchi, Y. Sakurai, T. Okano, *J. Biomed. Mater. Res.* **1999**, *45*, 355; b) K. Nishida, M. Yamato, Y. Hayashida, K. Watanabe, N. Maeda, H. Watanabe, K. Yamamoto, S. Nagai, A. Kikuchi, Y. Tano, T. Okano, *Transplantation* **2004**, *77*, 379.
[17] Y. Miyahara, N. Nagaya, M. Kataoka, B. Yanagawa, K. Tanaka, H. Hao, K. Ishino, H. Ishida, T. Shimizu, K. Kangawa, S. Sano, T. Okano, S. Kitamura, H. Mori, *Nat. Med.* **2006**, *12*, 459.
[18] T. Okano, N. Yamada, M. Okuhara, H. Sakai, Y. Sakurai, *Biomaterials* **1995**, *16*, 297.
[19] R. Yoshida, K. Uchida, Y. Kaneko, K. Sakai, A. Kikuchi, Y. Sakurai, T. Okano, *Nature* **1995**, *374*, 240.
[20] D.-S. Shin, J. Hyun Seo, J. L. Sutcliffe, A. Revzin, *Chem. Commun.* **2011**, *47*, 11942.
[21] L. Yang, F. Pan, X. Zhao, M. Yaseen, F. Padia, P. Coffey, A. Freund, L. Yang, T. Liu, X. Ma, J. R. Lu, *Langmuir* **2010**, *26*, 17304.
[22] G. Decher, *Science* **1997**, *277*, 1232.
[23] a) J. Schmitt, G. Decher, W. J. Dressick, S. L. Brandow, R. E. Geer, R. Shashidhar, J. M. Calvert, *Adv. Mater.* **1997**, *9*, 61; b) Z. Tang, Y. Wang, P. Podsiadlo, N. A. Kotov, *Adv. Mater.* **2006**, *18*, 3203.
[24] J. Chen, S.-W. Huang, W.-H. Lin, R.-X. Zhuo, *Small* **2007**, *3*, 636.
[25] J. Chen, X.-M. Xia, S.-W. Huang, R.-X. Zhuo, *Adv. Mater.* **2007**, *19*, 979.
[26] M. Matsusaki, K. Kadowaki, Y. Nakahara, M. Akashi, *Angew. Chem. Int. Ed.* **2007**, *46*, 4689.
[27] a) J. A. Burdick, K. S. Anseth, *Biomaterials* **2002**, *23*, 4315; b) J. B. Leach, K. A. Bivens, C. W. Patrick, C. E. Schmidt, *Biotechnol. Bioeng.* **2003**, *82*, 578.
[28] a) H. A. Awad, M. Q. Wickham, H. A. Leddy, J. M. Gimble, F. Guilak, *Biomaterials* **2004**, *25*, 3211; b) D. L. Elbert, C. B. Herbert, J. A. Hubbell, *Langmuir* **1999**, *15*, 5355; c) M. Y. Li, M. J. Mondrinos, M. R. Gandhi, F. K. Ko, A. S. Weiss, P. I. Lelkes, *Biomaterials* **2005**, *26*, 5999.
[29] a) P. T. Hammond, *Adv. Mater.* **2004**, *16*, 1271; b) S. S. Shiratori, M. F. Rubner, *Macromolecules* **2000**, *33*, 4213; c) F. Caruso, R. A. Caruso, H. Mohwald, *Science* **1998**, *282*, 1111.
[30] S. S. Ono, G. Decher, *Nano Lett.* **2006**, *6*, 592.
[31] T. Fujie, N. Matsutani, M. Kinoshita, Y. Okamura, A. Saito, S. Takeoka, *Adv. Funct. Mater.* **2009**, *19*, 2560.
[32] A. Banfi, A. Muraglia, B. Dozin, M. Mastrogiacomo, R. Cancedda, R. Quarto, *Exp. Hematol.* **2000**, *28*, 707.
[33] J. Sanchez-Ramos, S. Song, F. Cardozo-Pelaez, C. Hazzi, T. Stedeford, A. Willing, T. B. Freeman, S. Saporta, W. Janssen, N. Patel, D. R. Cooper, P. R. Sanberg, *Exp. Neurol.* **2000**, *164*, 247.
[34] A. R. Derubeis, R. Cancedda, *Ann. Biomed. Eng.* **2004**, *32*, 160.
[35] a) H. J. Jin, J. S. Chen, V. Karageorgiou, G. H. Altman, D. L. Kaplan, *Biomaterials* **2004**, *25*, 1039; b) P. Kasten, R. Luginbuhl, M. van Griensven, T. Barkhausen, C. Krettek, M. Bohner, U. Bosch, *Biomaterials* **2003**, *24*, 2593; c) J. R. Mauney, C. Jaquiery, V. Volloch, M. Herberer, I. Martin, D. L. Kaplan, *Biomaterials* **2005**, *26*, 3173.
[36] O. Frank, M. Heim, M. Jakob, A. Barbero, D. Schafer, I. Bendik, W. Dick, M. Heberer, I. Martin, *J. Cell. Biochem.* **2002**, *85*, 737.
[37] a) J. Park, J. Ries, K. Gelse, F. Kloss, K. von der Mark, J. Wiltfang, F. W. Neukam, H. Schneider, *Gene Ther.* **2003**, *10*, 1089; b) A. Winter, S. Breit, D. Parsch, K. Benz, E. Steck, H. Hauner, R. M. Weber, V. Ewerbeck, W. Richter, *Arthritis Rheumatism* **2003**, *48*, 418.
[38] J. Hong, B.-S. Kim, K. Char, P. T. Hammond, *Biomacromolecules* **2011**, *12*, 2975.
[39] a) J. Hong, N. J. Shah, A. C. Drake, P. C. DeMuth, J. B. Lee, J. Chen, P. T. Hammond, *ACS Nano* **2012**, *6*, 81; b) M. L. Macdonald, R. E. Samuel, N. J. Shah, R. F. Padera, Y. M. Beben, P. T. Hammond, *Biomaterials* **2011**, *32*, 1446; c) N. J. Shah, M. L. Macdonald, Y. M. Beben, R. F. Padera, R. E. Samuel, P. T. Hammond, *Biomaterials* **2011**, *32*, 6183; d) H. W. Jomaa, J. B. Schlenoff, *Macromolecules* **2005**, *38*, 8473.
[40] a) A. S. Badami, M. R. Kreke, M. S. Thompson, J. S. Riffle, A. S. Goldstein, *Biomaterials* **2006**, *27*, 596; b) B. G. Keselowsky, D. M. Collard, A. J. Garcia, *Biomaterials* **2004**, *25*, 5947; c) B. G. Keselowsky, D. M. Collard, A. J. Garcia, *Proc. Natl. Acad. Sci. U. S. A.* **2005**, *102*, 5953.