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Two dimensional nanosheets as conductive, flexible elements in biomaterials

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Since the discovery of graphene in 2004, two-dimensional (2D) nanostructures have been attracting tremendous interests for a variety of potentials including bio-inspired application due to their fascinating electronic, mechanical and optical properties. Especially, graphene and other 2D or quasi-2D nanostructures show excellent conductivity and flexibility. Here, we highlight recent impressive progress concerning the use of two representative types of 2D and quasi-2D nanostructures, graphene-based nanosheets and ultrathin polymeric nanosheets, as conductive or/and flexible elements for engineering the three dimensional (3D) tissues. The results have featured the unique potential in biomaterials of tissue engineering research field for such 2D and quasi-2D nanostructures.

Introduction

Since the discovery of graphene in 2004[1], nanostructures in the two dimensional form, which include while are not limited to graphene, have been attracting tremendous interests for a variety of uses including bio-inspired application due to their fascinating properties.[2-4] Specifically, graphene and/or its chemical derivatives, which are referred as graphene-based nanosheets below, have shown conductivity, flexibility and mechanical strength.[2-4] They also show the ability to support cellular proliferation, adhesion, and differentiation with little or no cytotoxic effects[5, 6]. In addition, graphene-based nanosheets also show high stability in aqueous solutions[7] and low inflammatory responses[8]. Combining with graphene's inherent properties of high conductivity with the electron mobility (up to 20,000 cm^2 V^{-1} s^{-1}) and flexibility[9, 10], such graphene-based nanosheets have been rapidly developing as promising candidates for various biological applications.

Except for the graphene-based nanosheets, ultrathin polymeric films composed of biodegradable polyesters such as poly(D,L-lactide) (PLA), poly(L-lactic acid) (PLLA), poly(glycolic acid) (PGA), and their copolymers are another interesting type of quasi-2D nanomaterials for the biological applications (referred as polymeric nanosheets below). They are a new class of soft nanomaterials, which are widely investigated in the polymer research field[11].Typical features of these polymeric nanosheets are tens to hundreds of nanometers thick with a very large aspect ratio (greater than 10^6), and unique physical properties such as large contact area, high levels of flexibility, noncovalent adhesion, selective molecular permeability, and heterofunctionality due to the surface heterogeneous functional ligands[12]. These properties enable the ultrathin nanosheets beneficial for the bio-inspired application.

The unique properties of such 2D or quasi-2D nanostructures enables their extraordinary potential for the bio-inspired application, especially tissue engineering. each year, there are enormous and increasing demands of organ transplantation for the patients who suffer from organ and tissue aging, damage or failure. To address this need, the field of tissue engineering, which creates transplantable tissues or organs to maintain, restore and even enhance nature tissue and organ function, has attracts a lively interest from biologists, materials scientists, engineers, and physicians. The principles of tissue engineering, which was firstly described by Langer and Vacanti early in 1993[13], offers potentially feasible solution to various diseases ranging from the orthopedic therapies to cardiovascular and diabetes diseases etc. Unfortunately, the current available tissues are still rare, which are only limited to those with simple tissue architecture and cellular organization such as skin epidermis, cartilage etc.[14], it is still challenging of engineering highly organized and functional 3D complex tissues for the clinical use, and this research field is currently under intense exploration.[15,16]

The native tissues and organs are composed of extracellular matrix (ECM), different cell types, and chemical and physical signalling cues.[15] Tissue engineering involves biomaterial scaffolds which serve as synthetic extracellular matrix to organize different cells into a specific 3D architecture to perform a specific function. The scaffold biomaterials are crucial for the development of tissue engineering, and it is challenging to meet the requirements for scaffold usage. Firstly, the scaffold micro-architecture should be modulated to be nano-fibrous to achieve biomimetic tissues, when considering the

collagen, the major protein of the ECM and controlling cell behaviour with its architecture, is nano-fibrous[17]. Secondly, the mechanical properties of the scaffold must be sufficient to withstand the mechanical stresses during tissue neogenesis[18]. Moreover, the scaffold biomaterials should be biocompatible to avoid an immune response, immunogenicity or cytotoxicity[19]. Additionally, scaffold should be also highly porous to allow efficient mass transport.[20] More importantly, conductivity and flexibility are crucial for the specific tissue regeneration, which imposes more difficulty for the choice of scaffold biomaterials. For example, the regeneration of a nerve tissue such as the spinal cord in neural tissue engineering requires anisotropic conduction within the cell-seeded construct, thus conductivity of scaffolds should be fulfilled. Also, for the local delivery of an engineered tissue in clinical therapies, scaffold must be sufficiently flexible to be aspirated and injected through a conventional biocompatible device. Because the commonly used scaffold materials are naturally non-conductive and/or rigid, recent studies suggest that the incorporation of nanomaterials as conductive and/or flexible elements is a rational and feasible approach to compensate these scaffold limitations.

2D or quasi-2D nanostructures developed in recent years hold the potential for engineering highly organized and functional 3D complex tissues, and have a significant impact on tissue engineering application. This article highlights the impressive research advances of graphene-based nanosheets and ultrathin polymeric nanosheets usage in the engineered 3D tissue constructs. Considering the 3D micro-environment represents the natural tissue micro-environment, the performance of these 2D and quasi-2D nanosheets in the 3D micro-environment is of realistic values to develop mimetic structures for the clinical therapies.

Graphene-based nanosheets as conductive or mechanically enhancing elements

Graphene, which is a single atomic layer of sp2 hybridized carbon atoms, has been the focus both in the academic research and industrial application due to its unique electronic structure and fascinating electronic, mechanical, and optical properties.[1, 9, 10,21] Meanwhile, a large number of graphene-derivatives have been reported through surface chemical modification by grating various chemical functional groups.[22-24] These novel graphene-based 2D nanosheets show remarkable features for their bio-inspired application. For example, they have shown the ability to support cellular proliferation, adhesion, and differentiation with little or no cytotoxic effects. [5, 6] They also exhibit high stability in aqueous solutions[7] and low inflammatory responses[8]. Combining with the inherent properties of high conductivity and flexibility [9, 10], such graphene-based nanosheets are prospective candidate to mimic certain structures and functions for tissue engineering application.

One of the impressive potentials for such graphene-based nanosheets lies in that they can be incorporated into matrix scaffold as conductive and/or mechanically enhancing elements to mimic the characteristics of native tissues. For such purpose, graphene oxide (GO) is preferred over graphene for preparing homogeneous aqueous suspensions due to the presence of oxygen-containing hydrophilic groups to reduce the irreversible agglomeration. Shin et al has incorporated graphene oxide (GO) into gelatin methacrylate (GelMA) for the creation of cell-laden graphene embedded hydrogels.[25] In their study, GelMA was chosen as the matrix scaffold due to its excellent photo-patternable properties to allow the fabrication of required microscale structures, together with the specific ability of exfoliating graphene from their bulk materials in an aqueous phase.[26, 27] The obtained hybrid hydrogels show tunable mechanical strength and enhanced electrical properties, which fulfills the requirement of scaffolds in tissue engineering application. Meanwhile, such hybrid hydrogels also exhibit the ability of supporting cellular spreading and alignment with improved viability and proliferation in a 3D micro-environment, which more accurately represents the natural tissue micro-environment compared to two dimensional (2D) systems. Compared to the CNT-embedded GelMA hybrid hydrogels and pristine GelMA hydrogel scaffolds reported before,[28, 29] such GO-embedded GelMA exhibited improved physical and biological properties due to the incorporation of GO, and the improved ability of faster cell attachment, spreading and elongation, which indicates the graphene-based nanosheets can be efficiently conductive and mechanically enhancing elements in the scaffold biomaterials. Importantly, we would like to highlight the advantages of GO-GelMA hybrid hydrogels in the fabrication of multilayered hydrogel structures with controllable thickness and mechanical properties, which has the potential to mimic the complex multicellular and stratified native tissues such as skin and blood vessels[30, 31]. As shown in **Figure 1(a)**, the authors fabricated three different types of double-layer constructs comprising of GelMA/GelMA, GO-GelMA/GelMA and GO-GelMA/GO-GelMA as the top layer/bottom layer. Overall, they found that the cell survival in the bottom layer was significantly improved when GO-GelMA replaced pure GelMA as the top hydrogel layer **(Figure 1(b-d))**, which is attributed to the protective effect of GO during the fabrication. Such results reveal more complex multilayer constructs can be made with the incorporation of GO.

However, the influence of the graphene-based nanosheets on cellular behaviors, which is important while still not clear for their usage as conductive and/or mechanically enhancing elements in scaffold biomaterials. It still remains a challenge to regulate the cellular behaviors on graphene-based nanosheets due to the complexity of cell responses and surface states of graphene-based nanosheets. Shi et al has proved that it is feasible to regulate the cellular behaviors on few-layer reduced graphene oxide (FRGO) films by controlling the reduction states of GO, especially the surface oxygen content of FRGO.[5] Such result gives more support of the potential of graphene-based nanosheets as conductive and/or mechanically enhancing elements in scaffold biomaterials. Ku et al. have investigated the cell adhesion, proliferation, and differentiation of mouse

myoblast C2C12 on unmodified, GO-, and reduced graphene oxide (rGO)-modified glass substrates, indicating myogenic differentiation was remarkably enhanced on GO substrate resulted from the physic-chemical properties control.[32] Recently, Ahadian et al indicate the electrical stimulation will significantly enhance myoblast cell differentiation on a thermally reduced graphene (TR)-Graphene substrate compared to GO and glass slide surfaces[33], revealing the cellular behavior of electrically active cells on graphene-based nanosheets can be modified by controlling the conductivity of graphene-based nanosheets. Such investigations prove that the cellular behaviors are surely influenced by the graphene-based nanosheets, indicating more biocompatible advantages of graphene-based nanosheets for the usage in scaffold biomaterials.

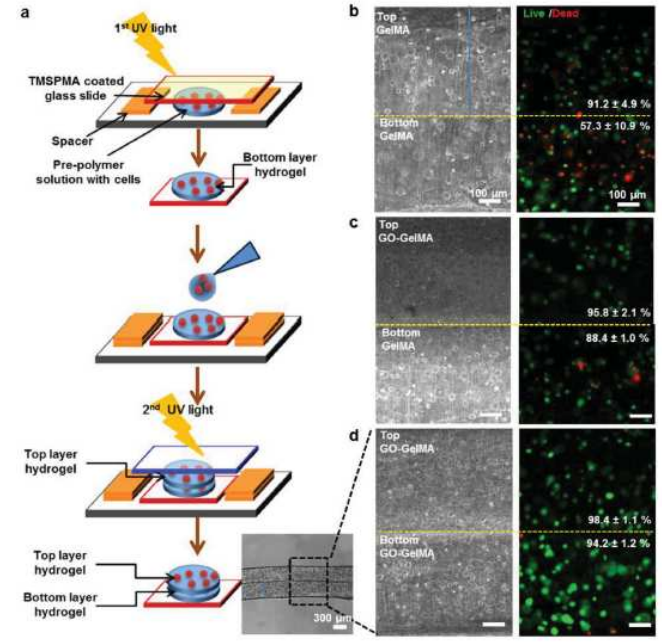

Figure 1. Schematic show of fabrication process (a) and the characterization of multilayer cell-laden hydrogel constructs (b-d). Reproduced with permission from ref 25. Copyright 2013, John Wiley & Sons.

The graphene-based nanosheets can be also used as the interlayer spacer except for the usage in scaffold biomaterials for the 3D tissue construction. Recently, Shin et al. have reported the development of multilayer cardiac tissue using a layer-by-layer (LbL) assembly technique by alternative cardiomyocytes cell seeding and poly-L-lysine (PLL) coated graphene oxide sheets (PLL-GO) nanosheets deposition on the GO-embedded GelMA hybrid hydrogels scaffolds.[34] As discussed above, GO-embedded GelMA substrate not only provides high electrical conductivity and mechanical properties, but also exhibits enhanced viability, elongation and proliferation of cells due to the GO incorporation when compared to those cultured on pristine GelMA. Meanwhile, the tissues were fabricated by using layer-by-layer (LbL) assembly technique, which has been verified to be feasible of engineering the multilayer tissue constructs with well-controlled cellular type and location such as blood vessels, skeletal muscle etc before.[35, 36]. We would like to highlight that the authors have employed the PLL-GO nanosheets as interlayer spacers with the consideration of excellent cell adhesive properties of PLL[37] and high conductivity and mechanical strength of GO, aiming to facilitate cell separation and stacking for the engineered dense and highly organized 3D complex architectures. As shown in (**Figure 2 (a) and (b)**), spatial organized multilayer cardiac tissues were successfully fabricated by using the LbL assembly of cardiac and endothelial cells (ECs) with PLL-GO thin films as interlayer spacers on GO-embedded GelMA scaffold. The fabricated tissues show well-developed and interconnected sarcomeric structures (**Figure 2(c)**), and are robust enough to be peeled off from the glass substrate (**Figure 2(d)**). Importantly, the tissues have demonstrated strong spontaneous and synchronous beating behavior (~20 or 30 beats per min (BPM)) only after one day of culture, and keep such strong spontaneous synchronous beating immediately after the detachment. Also, this frequency-dependent actuation can be controlled by applying a low external electric field (1.4 V/cm), as shown in **Figure 2 (e),** suggesting the multilayer 3D cardiac tissues with PLL-GO thin films as interlayer spacers have great potential as biohybrid actuators. The current results indicate that the PLL-GO nanosheets embedded within the multilayer cardiac constructs improved cardiac cell organization, maturation, and cell-cell electrical coupling. Therefore, graphene-based nanosheets show their unique advantages in the fabrication of electrically propagating complex tissues, not only as scaffold biomaterials, but also interlayer spacer.

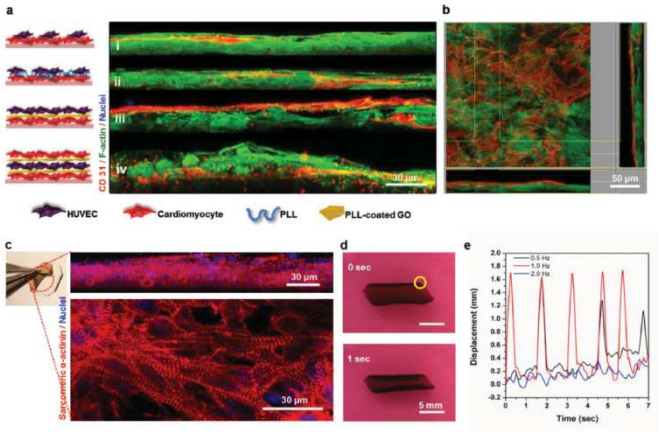

Figure 2. (a) Schematic show (left) and characterization (right) of 2L cardiomyocytes and ECs, (i): without any ECM layers, (ii): with pristine PLL, (iii): with PLL-coated GOs, and (iv): 3L cardiomyocytes and ECs with PLL-coated GOs as the interface layers between cells. (b) Widespread EC networks of sample (iii). (c) The 2L cardiomyocyte construct after 3-days of incubation shows the interconnected sarcomeric structures (top image: cross-section; bottom image: top view). (d) Optical images of the 2L cardiac actuator, and the displacement over time under electrical stimulation with the focus on the tip inside

the yellow circle were shown in (e). Reproduced with permission from ref 34. Copyright 2014, John Wiley & Sons.

Graphene-based nanosheets exhibit fascinating properties including conductivity, flexibility and mechanical strength. They also show the ability to affect the cellular behaviors such as cellular proliferation, adhesion, and differentiation. Recent research efforts give strong and impressive experimental evidences for their usage as conductive and mechanically enhancing elements in biomaterials for the tissue engineering application.

Ultrathin polymeric nanosheets as flexible element

Except for the graphene-based nanostructures, ultrathin polymeric films in 2D form (referred to the nanosheets), which range from tens to hundreds of nanometers thick with a very large aspect ratio (greater than 10^6), also attracts interests for the bio-inspired application. Unlike bulk polymeric films, such polymeric nanosheets have unique physical properties, which are beneficial for their biological applications. For example, their thermodynamic and mechanical properties vary in a thickness-dependent manner, resulting in high flexibility, non-covalent adhesiveness, and selective molecular permeability.[12] Furthermore, the surface property of these ultrathin polymeric nanosheets could be tailored by integration of nanoparticles[38] or nanotubes.[28]

One unique feature of such ultrathin polymeric nanosheets lies in its flexibility, which could assist in the engineering of some specific biomimetic tissue structures.[39] For example, it would be an ideal platform for the local delivery of a cultured retinal pigment epithelial (RPE) cells monolayer on them. Medical researchers believe the development of subretinal choroidal neovascularization, which is caused by an increment in secretion of vascular endothelial growth factor from RPE cells, might be the pathogenesis of age-related macular degeneration (AMD).[40] Therefore subretinal transplantation of RPE cells to the site of degeneration would offer an ideal treatment in the clinical therapies. Unfortunately, the transplantation of autologous peripheral RPE cells has been proved to suffer from the low viability of the injected cells, restricted distribution and integration into the subretinal tissue[41], thus an alternative approach which involves the local delivery of an engineered RPE monolayer has attracted increasing attention in recent years. We would like to highlight the recent research advance of a magnetic nanoparticles-embedded biodegradable poly(lactic-co-glycolic acid) (PLGA) micropatterned nanosheets reported by Fujie et al, which can support a stable monolayer of RPE cell line (RPE-J cells) growth.[42] Owing to the high flexibility of the nanosheets, the RPE monolayer could be injected through a clinical syringe without significant loss of cell viability. In contrast, common rigid substrates such as collagen, poly(ethylene terephthalate) and poly(methyl methacrylate)[43-45], which are typically micrometers in thickness and several millimeters in size, cannot be injected through a conventional syringe needle into the narrow subretinal space thus requires large incision. Therefore, such extremely flexible scaffold can avoid leakage of vitreous fluid and postsurgical infection caused by large incision, and holds notable potential for the invasively delivery of the engineered RPE monolayer. Impressively, the authors have demonstrated the delivery of the micropatterned nanosheets to the subretinal space of a swine eye *ex vivo* in order to evaluate the feasibility of the clinical therapies. As shown in **Figure 3 (a) and (b)**, the fabricated polymeric nanosheet was shown to be successfully injected and spread into the subretinal space, then attached to the macula and retained its circular shape after removal of the saline, which was partially filled before injection to secure the transplanted site. The RPE monolayer was also indicated to be stable inside the catheter needle and retained cellular activity (**Figure 4 (c)**). Therefore, these results *ex vivo* indicate a substantial potential of using micropatterned ultrathin polymeric nanosheets for delivering an engineered RPE monolayer in a clinical AMD therapies due to its unique flexibility.

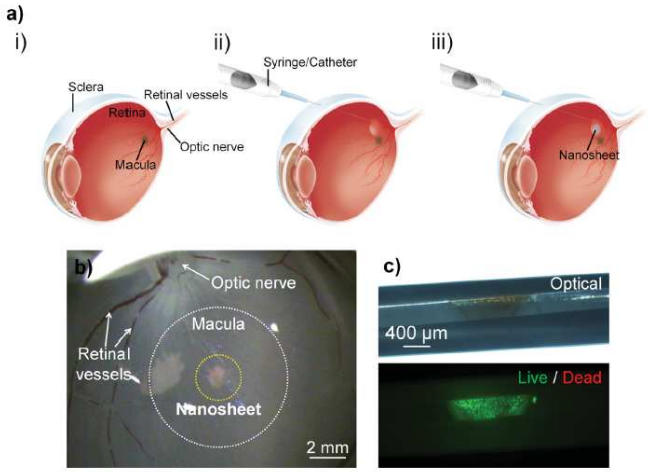

Figure 3. (a) Schematic of the injection of a micropatterned nanosheet into the subretinal space of a swine eye *ex vivo*. (b) Microscopic image showed the injected nanosheet was fixed on the macula after removal of the saline. (c) The micropatterned nanosheet with live/dead RPE-J cells in the catheter. Reproduced with permission from ref 42. Copyright 2014, John Wiley & Sons.

Another important and impressive instance of the 2D polymeric nanosheets as flexible element is to fabricate the periosteum-mimetic structures. Periosteum, which is a thin membrane that encases the surfaces of most bones, provides mechanical support to tendons. Its inner cellular layer, which possesses an exquisite topographic surface that consists of longitudinally oriented cells and collagen fibers, plays a key role in bone regeneration.[46, 47] The potential of the periosteum to harness and regulate cell arrangement, collagen fiber alignment, and the direction of bone development has been verified *in vitro* and *in vivo*, and research efforts have conducted to construct engineered periosteum using collagen and hydrogels.[48-50] However, one distinct disadvantage of such artificial periosteum films exists in the fact that they are thick and are difficult to effectively anchor to or integrate with bone

scaffolds, which will lead to serious complications during clinical interventions. Therefore, ultrathin polymeric nanosheets may be a promising candidate for generating artificial periosteum due to its unique feature of flexibility.

Recently, Shi et al has developed flexible "sticker-like" poly(lactic-co-glycolic acid) (PLGA) microgrooved nanosheets, for regulating cell and ECM arrangement in a manner similar to natural periosteum.[51] Such PLGA nanosheets were fabricated by a combination of spin coating and micro-patterning technique, and were verified to be noncovalently adhered onto various scaffolds and implants including chicken humeral bone, a macroporous hydroxyapatite/β-tricalciumphosphate (HA/β-TCP, the average pore size was 200–400 μm and the average porosity was 83%) scaffold, microporous β-TCP scaffold (pore size was less than 20 μm), and titanium block with a high stability and adhesiveness in aqueous conditions. The cell alignment of the stem cells cultured on them was shown to be effectively regulated. More impressively, the osteogenic differentiation of stem cells on the titanium blocks covered with microgrooved nanosheets has exhibited very interesting results. As shown in **Figure 4 (a)**, the microgrooved nanosheets-covering titanium blocks exhibit a higher levels of Runx/Cbfa-1 (an early osteogenic marker that binds specific DNA sequences that activate the development of stem cells into preosteoblasts) and SPARC (also termed osteopontin, an important protein for regulating bone mineralization and bone remodeling) compared to the flat PLGA nanosheet-covering titanium block and bare titanium block after 14 days of culture. Results of quantitative reverse transcription polymerase chain reaction (qRT-PCR) to amplify the expressed transcripts from the osteogenic genes (ALP, Runx2/Cbfa-1, type I collagen, and osteocalcin) (**Figure 4 (b)**) also indicate cells on the microgrooved nanosheet-covering titanium block exhibited significantly higher osteogenic commitment due to the strong expression of Runx2/Cbfa-1, type I collagen (the main template for mineralization and the most abundant protein in bone), and osteocalcin. Such results suggest that the microgrooved PLGA nanosheet directed not only cellular alignment but also protein and gene expression levels, which is an ideal mimic of the periosteum-like structure. The development of periosteum biomimics with 2D polymeric nanosheets may provide an innovative strategy for bone regenerative therapies.

Overall, similar with graphene-based nanosheets, ultrathin 2D polymeric films have also exhibited their unique properties which are beneficial for their bio-inspired applications. They are promising candidates to mimic certain structure and functions for tissue engineering, such as ideal scaffold for the local delivery of a cultured retinal pigment epithelial (RPE) cells monolayer on them, and artificial periosteum due to its unique feature of flexibility.

Conclusions

Two Dimensional or quasi-2D nanostructures have been attracting many interests for a variety of bio-inspried applications as conductive or/and flexible elements. The recent progress of graphene-based nanosheets and ultrathin polymeric nanosheets has shown great potential to engineer tissues, mostly *ex vivo*. The results suggest such 2D or quasi-2D nanosheets have been rapidly developing as promising candidates as conductive and mechanically enhancing elements in scaffold biomaterials, as interlayer spacer for the 3D tissue construction, and to mimic certain structure and functions in tissue engineering research field. However, there are still many challenges for these 2D or quasi-2D nanosheets before they offer feasible clinical solution to various cases. The long term toxicity of graphene- and polymeric-based nanosheets to cells is still elusive. More studies *in vivo* are highly desired to address the issues that may come from the complication environment *in vivo*.

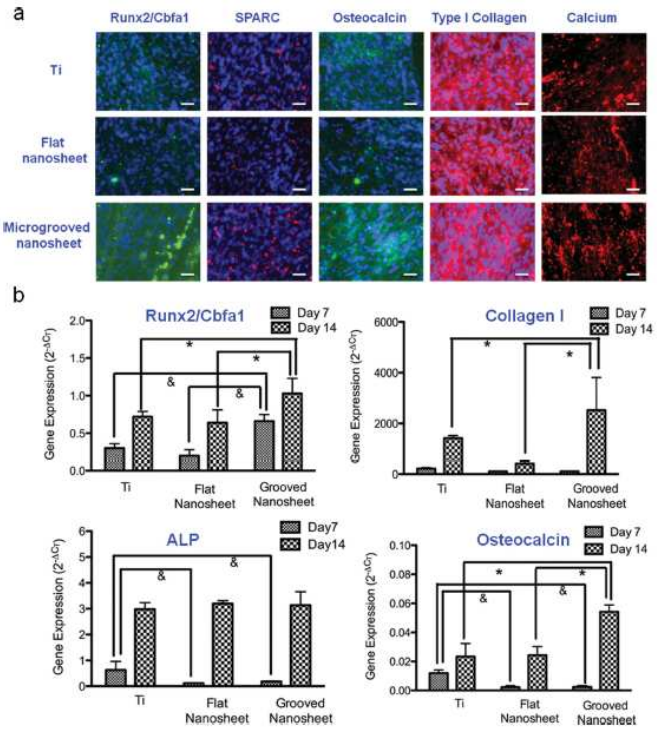

Figure 4. Immunofluorescent (a) and Gene expression (b) of stem cells cultured on microgrooved PLGA nanosheet-covering titanium blocks, flat PLGA nanosheets-covering titanium blocks, and bare titanium blocks for 14 days. & and * indicate statistical significance when compared with cells on the titanium block and microgrooved nanosheet-covering titanium block at 95% confidence level. Reproduced with permission from ref 51. Copyright 2014, John Wiley & Sons.

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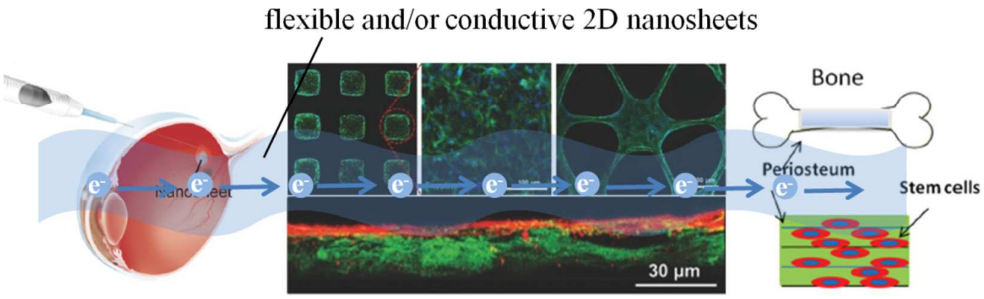

Two dimensional nanosheets have great potentials as conductive and/or flexible elements in biomaterials.