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Advances in Nanosheet Technology Towards Nanomedical Engineering

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Introduction

Nanobiotechnology plays an important role in the development of clinical therapeutics and diagnostics e.g., bioimaging/sensing,¹ drug delivery systems² and tissue engineering.³ Tissue engineering contributes to regenerative medicine, which generates tailor-made transplantable biological tissues by employing an engineered cellular matrix. However, this innovative approach also has some drawbacks such as an elevated risk of infection during cell processing and extended periods of cell culture. Thus, there are ongoing efforts towards the development of so called smart biomaterials that enhance the healing process in wound tissue or assist in the integration of implanted cells by directing cellular organization. Nonetheless, the engineered materials should be stable at the wound site without eliciting an inflammatory response or post-surgical adhesion. Thus, reducing the side effects of the implanted materials is crucial for the improvement of conventional therapeutics.

Ultra-thin polymeric films (often called as nanosheets, nanofilms or nanomembranes) are a new class of polymeric nanomaterials, conventionally studied in the field of polymer physics.⁴ These films are typically tens-of-nm in thickness and have unique interfacial and mechanical properties that are controlled in a thickness-dependent manner, resulting in non-covalent adhesiveness, tunable flexibility and molecular permeability (Fig. 3.1).^{5,6,7} In addition, a quasi-two-dimensional feature of the nanosheet is an attractive structure for synthetic mimics of extracellular matrix (ECM) in native tissues, which has an ideal structure and function to direct the cellular organization and therefore to regenerate and maintain tissues and organs. These properties are beneficial for the development of advanced biomaterials, including wound dressings, drug release devices and tissue engineering materials. In this chapter, we introduce recent developments in nanosheet technology for biomedical applications, focusing on the fabrication, physical properties and practical applications of nanosheets, particularly in the area of surgical procedures (e.g., wound dressing materials) and tissue engineering (e.g., cellular scaffolds).



Unique properties Flexible, Adhesive, Tailorable



Transferable to tissues & organs



Flexibly transformable structure under micropipette manipulation

FIGURE 3.1

Biomedical application of polymer nanosheets utilizing their unique characteristics such as physical adhesiveness and high flexibility. Nanosheets with tens- to hundres-of-nm thickness ($2 \text{ cm} \times 2 \text{ cm}$) were transferred to the human skin surface or manipulated by a micropipette, respectively (partially reproduced from references 7 and 34).

Fabrication and Fundamental Properties of Nanosheets

Preparation of freestanding nanosheets

There have been significant developments over the past five years in the fabrication of freestanding nanosheets. Typical characteristic features of the nanosheets are as follows: (i) a thickness of tens-of-nm, (ii) a huge size-aspect ratio (>10⁶), (iii) unique interfacial and mechanical properties, such as tunable flexibility, non-covalent adhesiveness and high transparency. From a structural viewpoint, a quasi-two-dimensional arrangement of polymer nanosheets could represent an ideal interface to mimic extracellular matrix (ECM) in biological tissues, which comprise of a well-organized permeable membrane that controls nutrient flux in living systems.^{8,9} Therefore, polymer nanosheets are regarded as a new category of quasi-two dimensional soft materials. Thus far, various techniques to fabricate the freestanding polymer nanosheets have been introduced, including a simple spincoating method, a Layer-by-Layer (LbL) method, a Langmuir–Blodgett method with crosslinkable amphiphilic copolymers and a sol–gel method with organic–inorganic interpenetrating networks.^{10,11,12,13}

One novel methodology for the fabrication of nanosheets is an LbL technique. The LbL method involves alternative deposition of oppositely charged polyelectrolytes by non-covalent bonding such as electrostatic interactions, hydrogen-bonding or hydrophobic interactions.^{14,15,16,17,18} Thus, a variety of functional electrolytes (including proteins, DNA or charged particles) can be integrated into the LbL structure. Applications of LbL-based nanomaterials have been explored in several fields such as electrochemical devices, chemical sensors, nano-mechanical sensors, nano-scale chemical/biological reactors and drug delivery systems.¹⁹ In particular, a combination of the spin-coating procedure with LbL (spincoating assisted layer-by-layer: SA-LbL) is useful for the preparation of well-organized nanosheets. The resulting material has a controllable thickness with a flat and smooth surface due to the high-speed horizontal diffusion of polymers during the spincoating process.

Freestanding polymer nanosheets can be detached from the solid substrate by employing a "sacrificial layer method" (Fig. 3.2a) or "(water-soluble) supporting film method" (Fig. 3.2b). In the sacrificial layer method, the precoated sacrificial polymeric layer is dissolved by specific organic solvents such as acetone or ethanol that do not dissolve the upper film. In the supporting film method, the water-soluble supporting film, such as a PVA film, is prepared on the surface of the SA-LbL film, which allows convenient collection of the free-standing nanosheet by peeling the complex film from an SiO₂ substrate, followed by dissolution of the PVA film. It should be noted that in a complex film, interaction between the nanosheet and the PVA film is greater than that between the nanosheet and the SiO₂ substrate. This transfer method is an efficient means of transferring the nanosheet from one substrate to another surface, including human skin or organs.



Preparation of freestanding polymer nanosheets by two different approaches: (a) sacrificial layer method and (b) supporting film method (partially reproduced from reference 6).

For example, we used polysaccharide electrolytes such as chitosan and sodium alginate, which have amino and carboxylic acid groups as cationic and anionic species at ambient pH (Fig. 3.3a). These polysaccharides are often used in biomedical fields, such as wound dressings or as artificial skin, because of their biocompatibility and bioabsorbability.^{20,21} Therefore, we prepared freestanding polysaccharide nanosheets by using the sacrificial layer method. Each polysaccharide layer was assembled on the sacrificial layer (e.g., cellulose acetate) by the SA-LbL method. The thickness was proportional to the number of layer pairs, suggesting a well-organized structure of the nanosheet at the nanoscale (Fig. 3.3b). The polysaccharide nanosheet was then released in acetone by dissolution of the cellulose acetate; the freestanding structure maintained the original size and shape of the SiO_2 substrate (Fig. 3.3c). Large-scale (90 μ m × 90 μ m) topographic images by AFM revealed that the surface of the polysaccharide nanosheet was as smooth and flat as the silicon wafer without any corrugations or wrinkles. From the cross-sectional analysis of the edge of the nanosheet, the AFM thickness of the nanosheet was estimated to be 30.2 ± 4.3 nm (10.5 layer pairs of polysaccharide), corresponding to the ellipsometric thickness (30.7 ± 4.5 nm) of the nanosheet on the SiO₂ substrate. As a result, the smooth and flat surface was obtained with root-mean-square roughness (RMS) of 7.1 ± 2.4 nm owing to the spincoating effect in LbL. It is noteworthy the same polysaccharide nanosheet can also be detached from the SiO₂ substrate by using the supporting film method, which can be then be released into water by dissolution of the PVA film.



Polysaccharide nanosheets: (a) Molecular structure of chitosan and sodium alginate, (b) thickness profile as a function of layer pairs, and (c) a freestanding polysaccharide nanosheet in acetone (partially reproduced from reference 34).

Poly(lactic acid) (PLA) is an aliphatic polyester made up of lactic acid (2-hydroxy propionic acid) building blocks.²² PLA has been widely studied not only because of its convenient production from lactic acid but also because of its biocompatibility and biodegradability, which make it a good candidate for biomedical applications.²³ In a similar manner to polysaccharide nanosheets, we also succeeded in fabrication of freestanding poly(L-lactic acid) (PLLA) nanosheets by both the sacrificial layer method and supporting film method. Unlike the LbL methods, these technique are applicable for various "hydrophobic" polymers, particularly biodegradable polyesters such as PLLA, poly(lactic-co-glycolic acid) (PLGA) and polycaprolactone (PCL). A PLLA solution was spincoated onto the PVA sacrificial layer on the SiO₂ substrate, or directly spincoated onto the SiO₂ substrate and subsequently detached using the PVA supporting film. The thickness of the nanosheet could be easily controlled because the thickness was proportional to the PLLA concentration used for spin-coating; the minimal thickness of the freestanding PLLA nanosheet was estimated to be 23 ± 5 nm when the concentration of the PLLA solution was 5 mg/mL. After dissolution of the PVA film, the freestanding PLLA nanosheet was obtained in water (Fig. 3.4a). The thickness was measured as 23 ± 5 nm and the RMS value was as low as 3.6 ±

0.5 nm. The PLLA nanosheet with an extremely high size-aspect ratio of greater than 10^6 maintained the same shape and size as the SiO₂ substrate (4 × 4 cm²). The transparent PLLA nanosheet could be scooped and held in air with a supporting wire frame (Fig. 3.4b), which was stable without bursting for at least one year.



FIGURE 3.4

PLLA nanosheets: (a) a freestanding PLLA nanosheet in water, (b) suspended by a wire loop (partially reproduced from reference 36).

Adhesive properties of the nanosheets

A micro-scratch test can be used to evaluate the macroscopic adhesive properties of ultra-thin films such as nanosheets.²⁴ The micro-scratch tester employs a diamond stylus that oscillates parallel to the surface of the nanosheet on the SiO₂ substrate. The adhesive failure of the nanosheet with the stylus is detected as the 'critical load' of the nanosheet, relative to the adhesive force. Interestingly, the critical load of the polysaccharide nanosheets drastically increased as their thickness decreased below 200 nm; the critical load of a 39-nm thickness nanosheet $(0.15 \times 10^6 \text{ N m}^{-1})$ was approximately 7.5 times greater than that with a thickness of 1482-nm $(0.02 \times 10^6 \text{ N m}^{-1})$ (Fig. 3.5a). Moreover, microscopic observations revealed different trail marks after scratching depending on the thickness of the nanosheet, such as 'cut-off (1482 nm)' and 'drawn (77 nm)'-like trails (Fig. 3.5a, inset). This observation suggested that the elasticity of the nanosheet was critically reduced at a thickness of less than 200 nm. In contrast, the critical load of the PLLA nanosheet (thickness: 23 ± 5 nm) was calculated to be 0.17 × 10^{6} N m⁻¹, and was equal to that of a nanosheet with a thickness of 60 ± 14 nm (0.18 × 10^{6} N m⁻¹) (Fig. 3.5b). These values were also comparable to that of a copper film (thickness: 200 nm, approximately 0.40×10^{6} N m⁻¹) on a glass substrate prepared by vacuum deposition under the same measurement conditions. However, when the thickness was over 100 nm, the critical load was significantly decreased. Furthermore, these values were the same as for the nanosheet fabricated directly on the SiO_2 substrate, indicating that the nanosheet with a large contact area could conform to the SiO_2 surface because of its exquisite flexibility and low roughness. Taking into account the results from the microscratching tests for both polysaccahride and PLLA, the adhesive properties generated by nanometeric thickness is of great potential in biomedical applications.



Adhesion properties of different nanosheets between (a) polysaccharide and (b) PLLA. Inset: microscopic morphologies of the polysaccharide nanosheets after performing the micro-scratch test for different thicknesses (1482 nm and 77 nm) (partially reproduced from references 35 and 36).

Mechanical properties of nanosheets

The bulge test, which vertically compresses a nanosheet placed on a plate with a circular hole (Fig. 3.6a), is frequently used for the evaluation of the mechanical strength of nanosheets.^{25,26} For example, three kinds of polysaccharide nanosheets with different thicknesses (35, 75 and 114 nm), were fixed on the steel plates with a 1 mm diameter circular hole in the center and kept under ambient conditions (temperature: $25 \pm 1^{\circ}$ C, humidity: $37 \pm 3\%$). It is noteworthy that the nanosheet adhered readily to the steel plate without using chemical adhesion. As pressure was applied to the polysaccharide nanosheet through the circular hole, deflection of the nanosheet was monitored from a side-view of the plates until distortion occurred (Fig. 3.6b). The relationship between pressure and deflection was non-linear, suggesting that the elasticity of the polysaccharide nanosheet was dependent on the total film thickness (Fig. 3.6c). The ultimate tensile strength (σ_{max}), elongation (\mathcal{E}_{max}) and elastic modulus (E) were calculated for nanosheets of different thickness from the initial elasticity of the stress-strain curve. The elastic modulus of the 35 nm polysaccharide nanosheet was 1.1 ± 0.4 GPa, which is considerably less than that of a cellulose film (E = 15 GPa) with a thickness of over 1 μ m. This result suggested that the nanosheet with a thickness of tens-of-nm is quite flexible due to its low elastic modulus. As the thickness of the polysaccharide nanosheet was increased, the elastic modulus increased to approach that of the bulk value (75 nm: 8.1 ± 2.5 GPa, 114 nm: 11.0 ± 1.6 GPa).



Mechanical properties of polysaccharide nanosheets: (a) bulge test apparatus, (b) nanosheets deflected by compressed air, and (c) pressure-deflection curve for different thicknesses of nanosheets (partially reproduced from reference 35).

Mechanical property of the PLLA nanosheet also shows similar trends. The PLLA nanosheet with thicknesses of 23 ± 5 nm deflected gradually and gave an almost semicircular deflection until a pressure of approximately 4 kPa was reached. The elastic modulus of the nanosheets was calculated to be 1.7 ± 0.1 GPa, which was quite low compared to the bulk PLLA (7-10 GPa).²⁷ Moreover, the mechanical properties of PLLA nanosheets were evaluated by means of "strain induced elastic buckling instability for mechanical measurement (SIEBIMM)".²⁸ The SIEBIMM test is based on the buckling metrology

between an elastic substrate (such as polydimethylsiloxane: PDMS) and the nanosheet under compression or stretching, which allowed calculation of Young's modulus of the nanosheet. The modulus is calculated by measuring the buckling wavelength of the nanosheet on a mechanically forced matrix. A continuous buckling pattern was clearly observed on the surface of the PLLA nanosheets after compression by PDMS strain relaxation (Fig. 3.7a). It is noteworthy that the wrinkle formation is no longer observed when the thickness was 703 nm due to the fact that the PLLA nanosheet was partially detached from the PDMS slab during the buckling process. This weak adhesiveness in higher thickness nanosheets could be explained by the thickness-related adhesion property of the polymeric nanosheets; increment of the nanosheet thickness reduced material flexibility as well as van der Waals force to the underlying materials. We previously evaluated the elastic modulus of PLLA nanosheets using the bulge test, although these measurements were only possible in the tens-of-nm thickness range due to low adhesion of nanosheets thicker than 100 nm. Considering the broader range of analyzed thicknesses, the SIEBIMM test was more suitable for evaluating Young's modulus of PLLA nanosheets. In fact, mean wavelength was proportional to the thickness of the PLLA nanosheets up to 318 nm (R^2 = 0.998) (Fig. 3.7b). The calculated Young's modulus of the PLLA nanosheet gradually increased as the thickness of the nanosheet increased (Fig. 3.7c). The Young's modulus of a 29-nm PLLA nanosheet is 3.5±1.3 GPa, while that of a 318-nm PLLA nanosheet is 6.6±1.7 GPa. Hence, we found that the mechanical modulus of both polysaccharide and PLLA nanosheets can be controlled by changing the thickness from tens to hundreds of nanometers.

Permeable properties of nanosheets

In general, PLA ($T_g^{\sim}58^{\circ}$ C) are classified as copolymers of poly(L-lactic acid) (PLLA) and poly(D,L-lactic acid) (PDLLA), which are produced from L-lactides and D,L-lactides, respectively. PLLA is known as a semi-crystalline polymer, which is rubbery above T_g and becomes a glass below T_g . By constrast, PDLLA is an amorphous polymer without crystallinity. In this regard, we focused on the crystalline domains of PLLA nanosheets by applying an annealing treatment above the T_g (referred as PLLA(+)), and envisaged using the resulting crystalline domains as a molecular sieve for a filtration membrane.²⁹ Atomic force microscope (AFM) images showed a remarkably morphological difference between PLLA (-) (Fig. 3.8a) and PLLA (+) (Fig. 3.8b), in which homogeneous distribution of grains attributed to PLLA crystals (~100 nm in diameter) as well as microscopic apertures between crystals (~100 nm in space) were clearly observed in PLLA (+) (Fig. 3.8c).





Mechanical properties of PLLA nanosheets with different thickness. (a) Optical images of buckled PLLA nanosheets by SIEBIMM test. (b) Measured wavelength. (c) Calculated Young's modulus as a function of film thickness. The shaded region in (c) shows the Young's modulus of a bulk PLLA film (partially reproduced from reference 9).

Thickness (nm)

Thickness (nm)



AFM images of 60 nm thick PLLA nanosheets before (a) and after (b) the annealing process ($80^{\circ}C$, 2 h), and (c) a magnified image of (b). Comparison of cumulative release of analytes through PLLA (+) (d and f) and PLLA (–) (e and g) as a function of different thicknesses (d and e, symbols in e) and different molecular weights (f and g, symbols in g). For (d) and (f), the thickness of the PLLA nanosheets was 60 nm (partially reproduced from reference 29).

The molecular permeability of the PLLA nanosheet was also evaluated as a function of the cumulative relase profile of analytes with respect to the film thickness and solution molecular mass of the analyte. The transfer of analytes through the nanosheet was continually monitored for up to 24 hrs using a UV-Vis spectrophotometer. A series of experiments were performed using model analytes of different

molecular weights. These model analytes included rhodamine B (RhoB: 479 Da / ~1.0 nm in size), vitamin B12 (VB12: 1,355 Da / ~2.4 nm), cytochrome C (Cyt C: 13.4 kDa / ~3.8 nm) and bovine serum albumin (BSA: 66.5 kDa / ~6.4 nm).³⁰ First, molecular permeability of RhoB was compared between PLLA (+) and PLLA (-) as a function of film thickness (60, 201, 314, 456 and 531 nm). PLLA (+) showed thickness-dependent permeability (Fig. 3.8d), while PLLA (-) displayed only a modest level of permeability (<10% after 12 hrs) for all thicknesses of PLLA (-) (Fig. 3.8e). Next, the molecular permeability through the 60-nm thick PLLA nanosheets was analyzed for different molecular weights of analytes; PLLA (+) displayed size-dependent permeability (Fig. 3.8f), while PLLA (-) showed slight permeability (<10% after 12 hrs) for all of the analytes (Fig. 3.8g).

Moreover, flux analysis indicated that the mass transport of PLLA (+) was controlled by the film thickness. Thus, modulation of the film thickness would give a critical threshold of molecular weight cut-off (MWCO) value for the filtration process. For example, MWCO of 60-nm PLLA (+) can be determined as ca. 10 kDa (less than 0.1 mmol $h^{-1}m^{-2}$ below MWCO). It is noteworthy that the selective permeability displayed by PLLA (+) was not evident in amorphous PDLLA. Therefore, the presence of PLLA derived crystalline domains in the nanosheet is crucially important for facilitating selective permeability. This technique is useful for the direct conversion of thermodynamic properties of semi-crystalline polymers to that of a nano-structured material e.g., selective molecular permeability.

Fabrication and Fundamental Properties of Nanosheets

Nano-adhesive plasters

Surgical repair for tissue defects is generally achieved by three fundamental methods; suture, plication and overlapping. Despite their high reliabilities for wound repair, the conventional repair of a tissue defect by suture and plication usually reduces the volume of the original tissue. For example, pulmonary air leakage due to visceral pleural injury is one of the most common postoperative complications after thoracic surgery. Plication of a pleural defect sometimes decreases respiratory function. Such complications might be caused by prolonged placement of a drainage tube and/or an extended period of hospitalization, which may even lead to thoracic empyema. Therefore, tight and firm repair of a pleural injury/defect is critically important in order to prevent air leakage.^{31,32} Nevertheless, it is sometimes difficult to suture or plicate a large defect or fragile tissue of the emphysematous lung. Overlapping is therefore seen as an ideal means of repairing a pleural defect, because it simply seals the injured surface without reducing the tissue volume of the injured lung. As a conventional sealant, fibrin glue (sheet) composed of fibrin-glue-coated collagen fleece, a typical adhesive material, is effective for the repair of a visceral pleural defect.³³ However, this material sometimes causes severe pleural adhesion. For high-risk patients with respiratory failure, such a severe pleural adhesion might further deteriorate pulmonary dysfunction, leading to serious complications. Therefore, a novel tissue sealant that does not cause tissue adhesion is required. Herein, we focused on the high flexibility and physical adhesiveness of the nanosheet. The flexible nanosheet can densely overlap and adhere to the biological surface like an adhesive plaster (e.g., human skin) (Fig. 3.1).³⁴ We envisage developing this concept further to generate a "nano-adhesive plaster" as a new class of wound dressing material. Such a material will overlap and treat the tissue defect in a minimally invasive way without an associated major inflammatory response or post-surgical adhesion.

Sealing operation using nanosheets

We employed a surgical procedure that involved using polysaccharide nanosheets to repair a visceral pleural defect in beagle dogs.³⁵ The polysaccharide nanosheet, or a fibrin sheet used as a positive control, was placed onto a pleural defect area prepared by a 3.2 cm² aorta punch on the right anterior, middle and posterior lobes. The 75 nm polysaccharide nanosheet was placed on a supporting PVA film (70 µm in thickness) for handling. By dissolving the PVA film with a PBS solution, the underlying nanosheet fitted on the curvature of the remaining tissue fully overlapping the pleural defect without any chemical adhesive reagents (Fig. 3.9a). After drying for a few minutes, the nanosheet was completely assimilated to the tissue surface. The airway pressure at which air leakage occurred, termed 'bursting pressure', was measured after repair using a manometer. The maximum airway pressure applied was 60 cmH₂O because air leakage could occur from the intact pulmonary hilum at higher pressures. At 5 min after repair, the nanosheet showed a bursting pressure $(31.7 \pm 10.3 \text{ cmH}_2\text{O})$ lower than that of the fibrin sheet ($45.0 \pm 5.5 \text{ cmH}_2\text{O}$). The bursting pressure of the nanosheet was slightly lower than that found in the bulge test (ca. 45 cmH₂O for the 6 mm diameter hole prepared on the steel substrate). At 3 hrs after repair, the outline of the square shaped nanosheet assimilating to the tissue surface could be faintly seen, and the bursting pressure of the nanosheet reached 56.7 ± 6.1 cmH₂O, which is the same level as that of the fibrin sheet.

From histological examination, it is noteworthy that the wound healing after treatment with the nanosheet was quite distinct from that with the fibrin sheet (Fig. 3.9b). Although it was difficult to observe the nanosheet overlap on the pleural defect, the formation of flat-shaped blood clots localized along the nanosheet was clearly observed in the region of the defect at 3 hrs after repair without significant inflammatory response. This finding suggested that blood cells initially deposited under the nanosheet were subsequently transformed to stable blood clots. At 3 days after repair, fibroblasts had grown around the blood clots, replacing the preformed clots. At 7 days after repair, angiogenesis was observed where the blood clots had originally formed under the nanosheet. Importantly, the sequence of the wound healing process never occurred on the outside of the polysaccharide nanosheet. Hence, no incidence of post-surgical adhesive lesion in the thoracic cavity was observed. At 30 days after repair, the original tissue-defect site was no longer discernible. In contrast to the polysaccharide nanosheet, repair of the pleural defect by the fibrin sheet exhibited large vacant air spaces at 3 hrs because the thick fibrin sheet was too firm to densely overlap the defect site. This lack of flexibility results in haphazard retention of blood components in the overlapped area. At 3 days, the random growth of fibroblasts was observed as well as the induction of an inflammatory tissue reaction, such as the emergence of macrophages. Furthermore, it is a critically important clinical issue that the fibrin sheet also strongly adheres to the chest wall. Severe pleural adhesions could reduce respiratory function and may cause a reoccurrence of pneumothorax.



(a) Schematic representation of visceral pleural defect repair using polysaccharide nanosheets, and (b) histological findings at different time points after treatment with polysaccharide nanosheets and fibrin sheet. (c) Macroscopic images of stomachs treated with PLLA nanosheet and conventional suture/ligation (partially reproduced from references 35 and 36).

PLLA nanosheets may be useful as dressing materials for acidic environments such as the stomach.³⁶ For example, an incision of approximately 1 cm in length was made in the anterior wall of the stomach in mice using a surgical knife. A supporting suture was stitched (without ligation) at the middle of incision line to invert the reflected mucosa. Thereafter, the PLLA nanosheet supported with the PVA supporting film (typically 1.5×1.0 cm) was placed over the incision site. Immediately after covering, the supporting suture (no ligation) was pulled out. The PVA supporting film was then dissolved in saline. At 7 days after surgical intervention (PLLA nanosheet- and suture-treated), the stomachs were removed from the mice. Sealing treatment with the nanosheet did not cause tissue adhesion, and surprisingly, few postoperative cicatrices remained on the surface of the stomach (Fig. 3.9c). In contrast, tissue adhesion was observed in several examples of suture-treated mice with apparent cicatrization in the stomach, causing severe deformity and shrinkage.

Histological observations also highlighted remarkable differences in wound healing between wounds sealed with a nanosheet or suture/ligation. In the nanosheet-sealed mice, the gastric mucosa at the incision site was loosely bent because the PLLA nanosheet just sealed the surface of the gastric serosa. Fibroblasts regenerating in response to wound healing grew normally and smoothly sealed the incision site; the thickness of fibroblasts was equal to that of serosa around the incision site. However, in the conventional suture/ligation-treated mice, gastric mucosa was tightly stitched by suturing. The number of regenerating fibroblasts markedly increased at the incision site, which is typical of the normal wound healing process following conventional suturing treatment. Our results suggest that the PLLA nanosheet directs the balance between conflicting phenomena involved in tissue repair and resistance to tissue adhesion. Specifically, when the surface of the PLLA nanosheet adheres directly to the stomach (obverse surface) it is exposed to blood and tissue fluid containing various growth factors. Fibroblasts grow normally on the surface of the PLLA nanosheet in the presence of growth factors. However, it is intrinsically difficult for cells to adhere to the outer surface of the PLLA nanosheet (reverse surface).

Overall, the nanosheets have desirable properties for acting as sealants in medical applications: high flexibility and physical adhesiveness without the requirement for chemical and biological adhesives used in conventional dressing materials. Thus, repair by overlapping a tissue defect with the polysaccharide nanosheet has significant advantages in maintaining the function of the remaining lung against sustained ventilation and the pressure from respiration and bleeding. Moreover, the PLLA nanosheet displays a sealing effect for a gastric incision procedure, which is restricted in conventional suturing surgery. It is also noteworthy that careful selection of polymers and related physical properties would be important for the application of nanosheets, depending on the types of tissue and organs.

Advanced therapeutics using drug loaded nanosheets

Bacterial infection is a major cause of peritonitis leading to severe sepsis. Postoperative anastomotic breakdown, which is one of the major complications after gastrointestinal surgery, also causes bacterial peritonitis.^{37,38} Therefore, therapeutic treatment by suture repair of a perforated/leaked lesion is crucial. Such procedures, however, are often technically challenging because the tissues in the area of the perforated/leaked lesion are usually inflamed and friable. A therapeutic approach for gastrointestinal perforation or high-risk/difficult anastomoses to replace conventional intervention is urgently needed. We reasoned that the polysaccharide nanosheet constitutes a stable platform for loading drugs such as antibiotics, which are an effective therapeutic tool against bacterial infection.



(a) Antimicrobial effect of polysaccharide nanosheets with (left) and without (right) tetracycline (TC) using the Kirby-Bauer assay. A clear zone of inhibition is seen around the nanosheet loaded with TC (left). (b) Macroscopic image of murine cecum treated with a PVAc-TC-nanosheet illuminated under black light. (c and d) Antiinflammatory effects of PVAc-TC-nanosheets for a period of 7 days after the operation for PVAc-TC-nanosheets (black circle), TC-nanosheets (gray circle) and PVAc-nanosheets (white circle). (c) Murine sviability and (d) number of bacteria in the intraperitoneal lavage. The dashed line in (c) represents murine viability for the sham (partially reproduced from reference 39).

We have developed an antibiotic-loaded nanosheet to inhibit bacterial penetration and investigated its therapeutic efficacy using a model of a murine cecal puncture.³⁹ Tetracycline (TC) was sandwiched between a poly(vinylacetate) (PVAc) layer and the polysaccharide nanosheet (named "PVAc-TC-nanosheet"). Under physiological conditions TC was released from the nanosheet for 6 hours. The antimicrobial effect of the PVAc-TC-nanosheet was evaluated by a Kirby-Bauer (KB) test.⁴⁰ Growth of *Escherichia coli* on the agar medium was inhibited by TC released from the PVAc-TC-nanosheet, but not by the PVAc-nanosheet (Fig. 3.10a). Hence, incorporation of TC in the nanosheet should show an antimicrobial effect. We optimized the amount of TC loaded on the nanosheet by varying the level of

antibiotic on a 1 x 1 cm² sized nanosheet. The size of the zone of inhibition (ZOI) plateaued above 8 μ g/cm² due to a saturating amount of TC diffusing into the medium. In general, a clinically recommended dose of TC determined by the KB test is 94 μ g/cm² (ZOI: 3.5-7.0 mm), which was calculated from the datasheet approved by the Clinical and Laboratory Standards Institute (CLSI).⁴¹ Hence, we determined the minimum loading amount of TC on the PVAc-TC-nanosheet as 6.2 ± 0.5 μ g/cm² (ZOI: 7.0 ± 1.7 mm). Thus, the dose of TC can be reduced by over 15 fold compared with the conventionally required dose. Such a reduction in the dosage of antibiotic should significantly reduce the incidence of adverse side effects in clinical practice.

A PVAc-TC-nanosheet, TC-nanosheet (without the PVAc layer) or PVAc-nanosheet (without the TC layer), each with the cut size of 1 cm \times 1 cm ([TC] = 6.2 μ g/cm²), was placed onto a cecal punctured lesion (0.8 mm²) contaminated with enterobacteria. All of the nanosheets were supported by the PVA film because the freestanding nanosheet itself shrank in air spontaneously. Thereafter, the supporting PVA film was dissolved by dropwise addition of a PBS solution, where the punctured lesion was sealed with a nanosheet in the absence of any adhesive agents. The punctured lesion covered with the PVAc-TC-nanosheet was observed upon illumination with black light. The results suggested a flexible and effective adhesion of the nanosheet on the murine cecum (Fig. 3.10b). In the sham group (without sealing), no mice survived longer than 5 days owing to lethal bacterial peritonitis (data not shown). It is noteworthy that the overlapping treatment with the PVAc-TC-nanosheet showed 100% survival in mice at 7 days, while the control TC-nanosheet and PVAc-nanosheet showed a survival rate of 55% and 45%, respectively (Fig. 3.10c, **p<0.01). Next, we examined the number of viable bacteria in murine peritoneal lavage one day after cecal puncture. Overlapping treatment with the PVAc-TC-nanosheet decreased the viable cell count by 15×10^4 -fold compared with the PVAc-nanosheet in the peritoneal lavage of the mice (Fig. 3.10d, *p<0.05). Our results strongly suggest that a PVAc-TC-nanosheet overlapping the punctured lesion affords significant protection against bacterial peritonitis by two distinct barrier effects. Firstly, a physical barrier caused by the nanosheet structure itself and secondly a pharmacological barrier due to the loaded antibiotic (TC). Thus, overlapping treatment with the PVAc-TC-nanosheet reduced the number of intraperitoneal bacteria as well as increasing mouse survival rate after cecal puncture. Taken together, these results suggest loading antibiotics on the nanosheet is an effective means of sealing the punctured lesion. Moreover, capping the surface of the nanosheet with a hydrophobic barrier, comprising a PVAc layer, is also important for the stable maintenance of the TC layer under physiological conditions. Hence, the PVAc-TC-nanosheet almost completely suppressed bacterial growth in the peritoneal cavity of mice with a cecal puncture, suggesting a complete inhibition of bacterial penetration through the PVAc-TC-nanosheet.

Various other drugs, including anticancer agents or growth factors, may be used in place of antibiotics in the drug layer of the nanosheet. For example, it is possible to embed anti-glaucoma drug (i.e., latanoprost) on the polysaccharide nanosheet. Indeed, the latanoprost-loaded nanosheet successfully down regulated intraocular pressure (IOP) reduction of rat cornea for 1 week.⁴² Therefore, integration of pharmaceutics will further enhance the applicability of nanosheets for advanced therapeutics; the nanosheet being an ideal platform to manage the controlled release of loaded drugs.

Patchwork coating by fragmented nanosheets

A burn wound is a complex and evolving injury. Extensive burn injuries produce, in addition to local tissue damage, systemic consequences.⁴³ In the management of burn wounds, much attention should be paid to minimize the risk of burn wound infection during wound healing. Otherwise, superficial and partial thickness wounds often deteriorate into deeper tissue damage. Severe sepsis resulting from burn wound infection is considered to be one of the most critical complications because of its

associated high mortality rate. A wide variety of wound dressings is currently available for the treatment of partial thickness burn wounds.⁴⁴ Although such conventional dressings appear to be suitable for wrapping relatively flat interfaces, it is often difficult to efficiently wrap burn wounds with an irregular (non-flat) shape such as those associated with fingers, toes and the perineum. To this end, we fragmented numbers of PLLA nanosheets into the suspended state, and performed a simple patchwork technique using the fragmented nanosheets to effectively wrap different shaped materials (Fig. 3.11a).⁴⁵ We investigated the coating properties of the nanosheet by first labeling it with octadecylrhodamine. Using a vertical dipping and lifting method, we were able to demonstrate that the labeled fragmented nanosheets efficiently coat several different interfaces, such as a lower half of the mouse body, including the perineum that constitutes an irregular shape, by fluorescence stereomicroscopy. The nanosheets were barely detectable under visible light, indicating that the ultrathin and flexible fragmented nanosheets could be adhered along the roughness of the interfaces at the nanometer scale. This is a noteworthy characteristic of nanosheets generated using the patchwork technique when adhered to an irregular surface.

We also studied an in vivo therapeutic barrier effect of the fragmented PLLA nanosheets using a mouse model of superficial dermal burn injury (SDB). Histological observations showed that the epidermis of the SDB-induced dorsal skin was defective by comparison with normal dorsal skin. Next, the suspension of the fragmented nanosheets was simply dropped onto the region of the SDB and then dried for 5 min. SEM observations clarified that the fragmented nanosheets could perfectly wrap the site of burn injury (Fig. 3.11b). This finding indicates that the flexible fragmented nanosheets adhere not only onto flat interfaces, such as SiO₂ substrate and membranes, but also onto uneven interfaces such as skin, resulting in a perfect patchwork. Next, we tested the effectiveness of the seal by carefully dropping a suspension of *Pseudomonas aeruginosa* onto the region of nanosheet-patchwork. We proposed the repeated patchwork treatment of the fragmented nanosheets as follows: SDB-induced skin was sealed with the fragmented nanosheets (1st patchwork). On day 3 after treatment of fragmented nanosheets, the region of nanosheet-patchwork was sealed or not with the nanosheets again (2nd patchwork), and then a suspension of *P. aeruginosa* was applied onto the same region. The repeated patchwork treatment of fragmented nanosheets was found to prevent the infection caused by degradation of the 1st patchwork. These findings suggest that the repeated patchwork treatment has the potential to prevent infection for longer periods of time (i.e., over 3 days). The patchwork technique using fragmented nanosheets shows immense potential as a novel burn wound therapy for both relatively flat dermal skin and skin with an irregular surface shape.



(a) A macroscopic image of fragmented PLLA nanosheets in water (left), on a SiO₂ substrate (center), and on murine skin (right, colored by rhodamine). (b) *In vivo* therapeutic barrier assay. (i) and (ii) SEM images of SDB-induced skin injury before (i) and after (ii) patchwork treatment with the fragmented nanosheets. (iii), iv) Histological images stained with H&E, showing the skin with SDB-induced injury without (iii) or with (iv) the nanosheet-patchwork. The letters A, D, FN, H, P, and S in the histological images indicate adipose tissue, dermis, fragmented nanosheets, hair root, P. aeruginosa and subcutaneous layer, respectively (partially reproduced from reference 45).

Tissue Engineering Applications of Nanosheets

Engineered interface for directing cellular organization

Directing cellular organization is important for the development of various synthetic tissues in biosensing, biorobotics and regenerative medicine. To this end, there have been significant efforts in recreating tissue structure by combining materials with nano- or microscale technologies.⁴⁶ ECM is made from nanofibrous structures (e.g., structural proteins and polysaccharides) containing numerous types of cell adhesive domains (e.g., collagen, laminin, fibronectin, vitronectin, and elastin). As such, ECM has an ideal structure and function to direct the cellular organization and therefore to regenerate and maintain tissues and organs (Fig. 3.12a). To mimic the ECM, topographically and mechanically tailored structures have been created by using polymeric materials, microfabrication techniques and functional nanomaterials (e.g., nanofibers, nanowires or nanotubes), which direct cellular organization and induce tissue formation.⁴⁷ Though materials, such as hydrogels and elastomers have been employed as cellular scaffolds owing to their tailorable structures and tunable mechanical properties, these materials display size and polymer components that sometimes hinder the hierarchical assembly of the cells into complex tissue structures. Thus, it is technically challenging to recreate the natural complexity of ECM in miniaturized engineered structures that aim to build functional tissue structures.

Microfabrication techniques to generate functional nanosheets

To engineer functional tissues in vitro, various novel approaches have been reported using micro- and nanostructured materials or cell manipulation techniques, such as porous polymeric scaffolds,⁴⁸ self-organized microwrinkles,⁴⁹ electrospun nanofibers,⁵⁰ bioprinting,⁵¹ and others.⁵² One such microfabrication technique (also known as "soft lithography"), which includes replica molding and microcontact printing, is a highly promising approach towards the creation of defined structures, shapes and arrangements at the micrometer scale. This technique employs microstructured elastomeric molds, consisting of poly(dimethyl siloxane) (PDMS), which allow for precise positioning of proteins and cells, control of shape and function of the cells, and even recapitulation of 3D culture microenvironments for highly structured cells and tissues.⁵³ One of the important achievements of soft lithography was microcontact printing (µCP); micropatterning of ECM molecules in a 2D configuration can display similar levels of tissue-specific differentiation in a 3D culture system. If ECM molecules are distributed as small, flat adhesive islands, such a configuration can control cell adhesion morphology in order to mimic characteristic cell shapes observed in native tissues. In this regard, we hypothesized that a quasi two-dimensional structure of free-standing nanosheets may be useful as synthetic mimics of the natural basement membrane in ECM, which has an amorphous, dense, sheet-like structure of 50-100 nm in thickness.⁵⁴ We attempted to recapitulate the ECM properties (such as flexibility, cell adhesiveness and nanostructure) on the nanosheets towards the development of functional nanosheets for use as flexible biodevices.



Biomimetic cellular organization directed by functional nanosheets: (a) Schematic representation of the ECM microstructure and (b) a functionalized nanosheet with cell-adhesive micropatterns. (c) A fluorescent image of fibronectin micropatterns. (d) Alignment of skeletal myoblasts (left) and myotubes (right) on PS nanosheets. (d) a macroscopic image of a freestanding nanosheet with micropatterned C2C12 myoblasts ($1 \times 1 \text{ cm}^2$). (e) A macroscopic image of rolled myoblasts on the nanosheet around a silicone tube ($3 \text{ mm}\phi$) (Inset: a cross-sectional image stained by calcein AM) (left), and a fluorescent image of the layered structure wrapped approximately twice around the tube (stained by CellTracker Green CMFDA) (right) (partially reproduced from reference 54).

We made freestanding nanosheets, and functionalized them with cell adhesive proteins by µCP for the anisotropic alignment of skeletal muscle cells (Fig. 3.12b). The alignment of the muscle cells is crucially important for their organization in muscle tissues. We employed polystyrene (PS) to generate the nanosheet, due to its manufacturability, well-known physical properties, ease of surface modification as well as its long history of use in cell culture applications. Prior to the spincoating of PS, we prepared

thermo-responsive sacrificial layer consisting of poly(*N*-isopropylacrylamide) (pNIPAM). The watersolubility of pNIPAM layer is drastically changed among lower critical solution temperature at 32°C. Thus, the PS nanosheet is stable at 37°C (pNIPAM: hydrophobic) during cell culture, and can be released at 4°C (pNIPAM: hydrophilic). Next, we prepared fibronectin (Fn) micropatterns on the nanosheet using μ CP to functionalize the film surface for organizing the cells. The μ CP process was performed by using poly(dimethyl siloxane) (PDMS) molds with microscopic groove-ridge features of 50 μ m in width and 50 μ m separation. These dimensions were chosen because it was shown that myotube alignment is enhanced on cell-adhesive micropatterns that are less than 100 μ m wide.⁵⁵ The unpatterned regions were rendered cytophobic by application of Pluronic F-127 to promote the initial cell alignment. The μ CP process resulted in the preparation of spatially controlled micropatterns on the nanosheet (Fig. 3.12c).

We also exploited the large surface of the nanosheet, and evaluated the effect of the Fn micropatterns on cellular morphology using murine skeletal myoblasts (C2C12). Surface structure is an important factor in directing the morphogenesis of myoblasts and myotubes. After 24 hrs of cell seeding, we observed the anisotropic alignment of C2C12 myoblasts on the Fn micropatterned surfaces (Fig. 3.12d). We also investigated myotube formation on the nanosheet because myotube alignment is crucial for maximizing the contractility of muscle tissue. After 8 days in differentiation medium, the formation of C2C12 myotubes was confirmed by immunostaining of myosin heavy chain. We observed aligned C2C12 myotubes on the Fn micropatterned surface. These findings suggested that the improved alignment of the myoblasts promoted end-to-end connection with each other, which favored the assembly of myotubes during the differentiation process.

Functional nanosheets towards flexible biodevices

Tissues with tubular structures, such as blood vessels and intestinal tracts, have a function that originates from their overall structure (e.g., controlled flux of blood or nutrients). In particular, the blood vessel has a specific structure consisting of multilayered smooth muscle cells with anisotropic alignment around the endothelialized layer. Thus, the recapitulated muscular structure may be a good model of the artery wall to study physiology and dysfunction of the blood vessels.⁵⁶ In this regard, the tubular structure mediated by the flexible nanosheet could be used for mimicking the natural tissue arrangement, which may facilitate the engineering of drug-screening devices. Thus, we utilized the freestanding nanosheets as an ultra-thin flexible substrate for building biomimetic cellular constructs. Specifically, we demonstrated how to generate an artificial tubular structure consisting of myoblasts cultured on the micropatterned nanosheet. These structures can be fabricated by simply rolling the cell/nanosheet construct around the template whilst maintaining cellular alignment. After one day of culture, we released the nanosheet bearing micropatterned myoblasts by dissolution of the pNIPAM layer at 4°C (Fig. 3.12e). Myoblasts subsequently aligned anisotropically along the CNT-Fn micropattern and remained viable. The freestanding cell/nanosheet construct was used to produce a tubular structure by wrapping it around a template (e.g., silicone tube, 3 mm diameter) (Fig. 3.12f). Although such a wrapping process to engineer multilayered tissue structures has been recently proposed,⁵⁷ they employed PDMS thin films that were more than 10 µm in thickness. As a consequence, there is always a thick barrier between the cells on the neighboring sheets. By contrast, the cross-sectional image of the rolled myoblasts on the nanosheet $(2 \times 2 \text{ cm}^2)$ showed a tightly wrapped structure surrounding the outer wall of the silicone tube (Fig. 3.12f, inset). From the lateral image, we also confirmed fluorescent signals of layered myoblasts due to the esterase activity of the myoblasts. The results suggest that the freestanding nanosheet can serve as a synthetic basement membrane to engineer hierarchical cellular organization. Moreover, the flexible nanosheet is such a spatially pliable structure that it can be shaped and integrated into a microfluidic system to study the functional properties of synthetic tissues.

Micropatterned nanosheets towards advanced cell delivery systems

There have been ongoing efforts towards the development of cell delivery systems to overcome several intractable diseases. Age-related macular degeneration (AMD) is the leading cause of visual impairment and blindness in the elderly population, whose main complication is the development of subretinal choroidal neovascularization and degeneration of retinal pigment epithelial (RPE) cells.⁵⁸ In this regard, subretinal transplantation of the RPE cells to the degenerated site has attracted a great deal of attention as an innovative therapeutic approach for the treatment of AMD.⁵⁹ However, poor viability, distribution and integration of the transplanted cells in suspension to the narrow subretinal space have limited this strategy. Therefore, the development of effective cell delivery devices would bring significant benefits for the treatment of AMD.

To this end, we focused on the high degree of flexibility of the nanosheets. Specifically, we designed micropatterned nanosheets consisting of biodegradable PLGA.⁶⁰ Next, the RPE monolayer was selectively engineered onto the micropatterned nanosheet to facilitate local delivery of the cellular organization to the narrow subretinal space in a minimally invasive way (Fig. 3.13a). Micropatterned nanosheets were prepared by a combination of spincoating and the μ CP technique. A PDMS stamp with columnar convex portions (diameter: 300-1000 µm) was fabricated by conventional photolithography using SU-8 molds. A PLGA solution was mixed with magnetic nanoparticles (MNPs) $(10 \text{ nm}\phi)$ in order to visualize the nanosheet, and the mixture was then spincoated onto the PDMS stamp. The resulting PLGA/MNPs layer was transferred onto a PVA coated glass substrate, on which collagen was spincoated to promote cell adhesion. Then, the sample surface was covered with RPE cell suspension, and the freestanding cell/nanosheet construct was obtained by dissolving the PVA layer in phosphate buffered saline (PBS). The PLGA/MNPs nanosheets with circular shapes were fabricated on the substrate, and dissolution of the PVA layer allowed for the release of brown colored nanosheets with 170 nm thickness (Fig. 3.13b, 500 μ m ϕ). Due to the high degree of flexibility, the freestanding nanosheet (e.g., 1000 μ m ϕ) was easily aspirated inside the intravenous catheter (24 G, 470 μ m in inner diameter) (Fig. 3.13c). Next, the RPE cells were selectively cultured on the micropatterned nanosheets. The cellular organization on the nanosheet was then characterized using a confocal laser scanning microscope (CLSM) because monolayer formation is an important structural aspect of epithelial cells. CLSM imaging clearly showed the RPE monolayer on the nanosheet (colored by Rhodamine B) (Fig. 3.13d).

Despite the mechanical share stress induced by aspiration and injection through the syringe needle, the RPE monolayer on the nanosheet retained its original shape without any fracture, and maintained >80% viability regardless of the sheet diameter. Moreover, we evaluated the thickness effect on cell viability after syringe injection. Finally, we demonstrated the injection of the micropatterned nanosheet to the subretinal space using a swine ocular globe, in which the freestanding micropatterned nanosheet (1000 μ m ϕ) was injected via an intravenous catheter. The injected nanosheet was successfully released and spread into the subretinal region where it subsequently fixed without structural distortion to the macula after removing the pre-filled saline (Fig. 3.13e). The flexible structure of the micropatterned nanosheet is beneficial not only for allowing deformation of the shape inside the needle, but also reducing the mechanical stress on the cell monolayer. This injectable micropatterned nanosheet that is delivered using a conventional syringe holds great promise for transplanting engineered cell monolayers in a minimally invasive fashion.

Conclusions and Future Outlook

In this chapter, we have described recent developments of nanosheet technology including the fabrication process, physical properties of the nanosheets themselves (structural, adhesive, mechanical and permeability) as well as their practical applications. The use of nanosheets as nano-adhesive plasters highlight the unique characteristics of these materials that make them ideally suited to surgical applications; in particular their high degree of flexibility and physical adhesiveness to tissue defects. Our *in vivo* results demonstrate that treatment involving the nanosheets is a minimally invasive procedure that does not elicit a significant inflammatory response. These benefits are crucial for designing implantable biomaterials. Further investigation of cell-material interaction involving the nanosheet is required in order to further analyze the surface properties of the nanosheet. In this regard, integration of advanced microfabrication techniques is an important approach to the understanding of the cell-material interface. Moreover, this methodology will be crucial for investigating how to direct cellular organization in tissue engineering applications. Nanosheets are an ideal platform for integrating various functions as exemplified by drug administration, development of new nanomaterials and even living organisms. We believe this research will open up new avenues for generating innovative biomaterials in the field of nanobiotechnology.

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Local delivery of retinal pigment epithelial (RPE) cells by micropatterned nanosheets: (a) schematic image, (b) a microscopic image of a micropatterned PLGA nanosheet (500 μ m ϕ), and (c) folded structure inside a 24 G intravenous catheter (470 μ m inner diameter). (d) A CLSM image showing monolayer formation by the RPE cells on the nanosheet (stained with rhodamine B), and (e) a microscopic image of the injected nanosheet, fixed onto swine macula (partially reproduced from reference 60).

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