Novel Polymeric Nanosheets for Gerontic Applications

Shinji Takeoka, Yoshihito Fukui, Toshinori Fujie, Yosuke Okamura

Abstract—**A polymeric nanosheet is a biocompatible sheet with nanometer thickness; e.g., one side is adhesive to skin or tissues and the other side provides a platform for various applications. We are proposing two kinds of polymeric nanosheets; one is a nanosheet with micrometer size prepared by thermal fusion of biodegradable PLGA nanoparticles adsorbed on the micropattern, and the other is a nanosheet with square centimeters size prepared by a layer-by-layer (LbL) technology with anionic and cationic polysaccharides. These nanosheets on the substrate were detached from the substrate in the treatment with sacrificial layers to provide the dispersion of the free-standing nanosheets. The micoscale nanosheets would be applicable to cure sclerosis or thrombosis because they have a recognition moiety for activated platelets. In the second type, we have demonstrated transference of the nanosheet from the silicone rubber substrate onto a skin surface or tissues. The invisible nanosheet on the skin or tissue will have obvious applications in the fields of skin-care or surgery. We are currently investigating the physical and optical properties of the nanosheets such as mechanical strength, structural colorization, biocompatibility and biodegradability and exploring gerontic applications.**

I. Introduction

In recent years, much attention has been paid to a minimal-invasive treatment in clinical aspects in the development of novel biomaterials. In drug delivery systems (DDS), vesicles, micelles, emulsions, and biodegradable nanoparticles have been extensively studied as carriers for biologically active substances such as drugs, recognition proteins, enzymes, genes, etc. [1]. We have developed biocompatible and biodegradable nanoparticles such as albumin-based nanoparticles [2-5] and vesicles [6, 7] carrying recombinant fragments of platelet membrane proteins [3, 4, 6, 8] and dodecapeptide (H12) derived from fibrinogen peptides as a recognition site for activated platelets [2, 5, 7, 9]. These nanoparticles specifically recognize the site of bleeding injury or activated platelets and show the hemostatic activity.

On the other hand, sheet-shaped carriers would have several advantages over spherical-shaped carriers such as a larger contact area for targeting, potential application of bilateral structures leading hetero-surface modification and unique dynamics caused of high flexibility than spherical carriers. The sheet-shaped carriers are expected to be conducted into DDS, to provide the suitable matrixes or scaffolds for platelet adhesion (Fig. 1.(a)). Contrastingly, they may cover pathological thrombus and prevent potential arterial sclerosis. Furthermore, the idea of sheet-shaped carriers can spread towards the construction of sealing biomaterials such as surgical wound dressing materials if the overall size of the nanosheets were quite large enough to cover the targeting skin or wound tissues

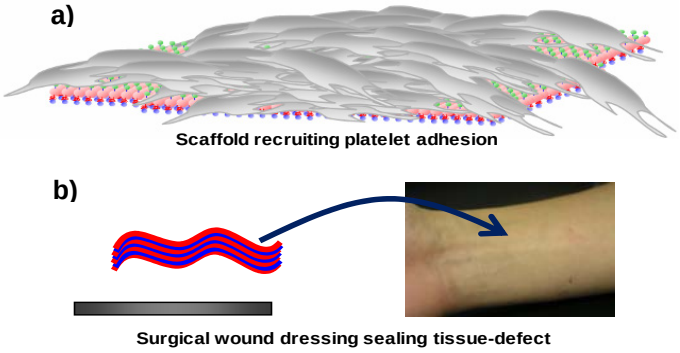

Fig. 1. Biomedical application of nanosheets: a) scaffold and b) dressing for wound treatment.

Key-techniques for construction of the sheet-shaped carriers are conventional surface technologies such as cast films [10], polyelectrolyte layer-by-layer (LbL) multilayers [11, 12], cross-linked amphiphilic Langmuir-Blodgett films [13], self-assembled monolayers (SAMs) [14], and assemblies of triblock copolymers [15]. Particularly, we focus on the methodology to simply prepare the free-standing nanoscale sheets such as SAMs and LbL .

In this paper, we are proposing two kinds of polymeric nanosheets; one is a nanosheet with micrometer size prepared by thermal fusion of biodegradable PLGA nanoparticles adsorbed on the micropattern, and the other is a nanosheet with square centimeters size prepared by a layer-by-layer (LbL) technology with anionic and cationic polysaccharides. These nanosheets on the substrate were detached from the substrate in the treatment with sacrificial layers to provide the dispersion of the free-standing nanosheets. Particularly, in the point of view for their clinical applications such as DDS in internal medicine and wound dressing in surgery, we describe that the one is an injectable nanosheet with the size and shape similar to native platelet in the concept of platelet substitutes, and the other is a giant nanosheet with a huge size-aspect ratio over 10^6, which can be transferred onto human skin or tissues, named nano-adhesive plasters, leading to gerontic applications.

II. Nanosheets with Micrometer Size from Nanoparticles Adsorbed on Micropattern

A. General Preparation of Nanosheets

Self-assembled monolayers (SAMs) have been widely applied to control physical and chemical properties of the

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surfaces of glass, quartz, SiO_2/Si wafers, or silica particles [16]. It is easy to construct micropatterned SAMs on silicon oxide using conventional photolithography processes [17]. The two-dimensional patterns have also been achieved in the particle array by site-selective deposition using chemical bonding or electrostatic interaction [18], an electrophotography method [19], a micromold method and gravity [20], a micromold method and a lateral capillary force [21], and a drying process of a colloidal solution onto the patterned Au film [22]. Furthermore, it was recently reported a novel method for deposition of a close-packed particle monolayer onto a patterned hydrophilic SAM [23]. However, there is no report on the preparation of free-standing nanoparticles-based nanosheets having uniform micrometer shape, nanometer thickness and heterogenous surfaces, for use as sheet-shaped carriers.

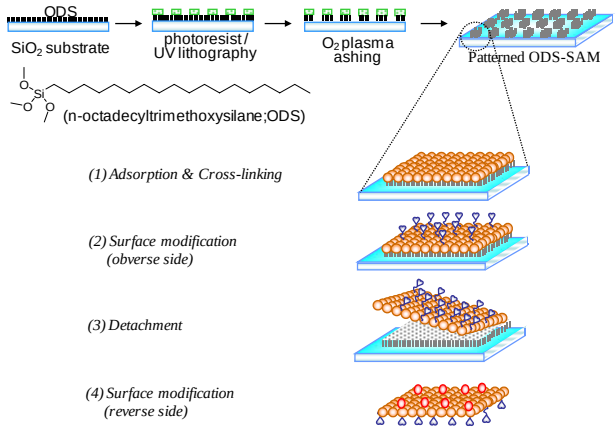

Fig. 2. Fabrication processes of free-standing nanosheets having hetero-surfaces.

Using the patterned SAMs as a template for fabrication of the nanosheet, we proposed a novel method to fabricate a free-standing nanosheet having hetero-surfaces by a combination of four processes in Fig. 2; (1) specific adsorption and two-dimensionally cross-linking of nanoparticles onto a patterned hydrophobic SAM region (ODS-SAM), (2) surface-modification (obverse side) of the resulting nanosheet, (3) cast a water-soluble sacrificial film to detach the nanosheets from the ODS-SAM, and (4) surface-modification (reverse side) of the nanosheet on the sacrificial film and dispersion by dissolution of the sacrificial film.

B. PLGA Nanosheets from PLGA Nanoparticles

A PLGA (lac/gly=85/15 w/w) ethyl acetate solution (2 wt%) was dropped in polyvinylalchol (PVA)/chitosan (2 wt%/0.3 wt%) mixed aq. solution and stirred at 1,000 rpm for 3 hrs, followed by homogenization at 42,000 rpm for 1 hr. After washing with distilled water, the PLGA particles coated with PVA and chitosan was collected by centrifugation (35,000g, 10 min, 4°C). The size of the resulting particles was 164±26 nm with high dispersibility and avoidance of non-specific binding. The amino groups of the coating chitosan were used to conjugate biomolecules with amide linkage. A conventional dry patterning process was adopted for the specified adsorption of the PLGA particles onto the patterned ODS-SAM (10 x 30 or 5 x 10 μm) as follows. When the dispersion of the PLGA particles (1.0 x 10^{11} /mL, pH 7.0) was applied to the substrate and the dispersion remaining on the substrate was then slowly blown off with a horizontal stream of N_2 gas, then the nanoparticles were arranged as a monolayer on the entire substrate, regardless of hydrophobic and hydrophilic regions. Next, the immediate and repeated washing of the substrate with a phosphate buffer (pH7.0) detached the nanoparticles from the hydrophilic SiO_2 region and the particles remained on the rectangular patterns. This indicated that the nanoparticles were firmly adsorbed onto the patterned ODS-SAM by hydrophobic interaction, whereas the nanoparticles on the SiO_2 region were weakly attracted with the hydrophilic region. After repeating the adsorption of the nanoparticles on the substrate and washing, the nanoparticles were closely packed in a monolayer pattern. The nanoparticle-adsorbed substrate was heated at 60°C (Tg of the particles) for 2 seconds on a hotplate after drying with N_2 to prepare nanosheets. A PVA film was cast on the entire surface of the substrate, and peeled off from the substrate as a transparent film. Observation of the PVA film using an SEM confirmed that the nanosheet was completely transferred to the PVA film as shown in Fig. 3.(a), and no residual sheets were observed on the substrate in good agreement with Stroock *et al* [24]. Such complete transfer should be possible because the hydrogen bonding between the PVA and the PVA/chitosan coated nanoparticles would be stronger than the van der Waals interaction of the dried nanosheets with the CH_3-terminal SAMs. Though the obverse surface of the resulting nanosheet was rough, remaining the spherical configuration of the constituent nanoparticles (Fig. 3.(b)), the neighboring nanoparticles were sufficiently fused to show a rectangular shape. When the nanosheet was dispersed from the ODS-SAM substrate using a PVA cast film as a sacrificial film, it was confirmed that the reverse side of the nanosheet, which was directly adsorbed on the ODS-SAM, was flat and smooth (Fig. 3.(c)), reflecting the surface morphology of the substrate. Furthermore, it was easy to dissolve the resulting PVA film to release the transferred nanosheet in a phosphate buffer at pH 7.4, and the free-standing nanosheets were collected on the membrane filter as shown in Fig.3.(d, e).

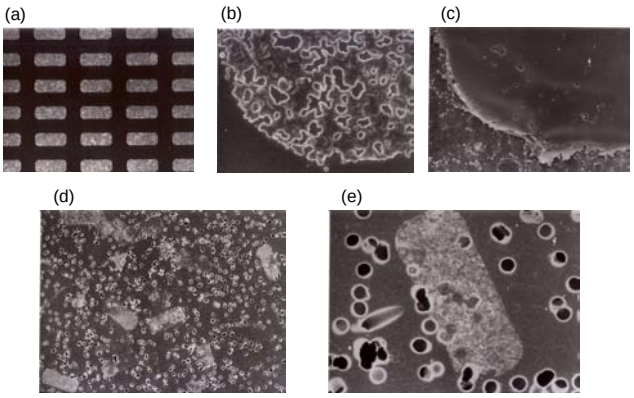

Fig. 3. Rectangular nanosheets (10 x 30 μm) transferred on the PVA film observed from the obverse side (a), the magnified edge part of the obverse side (b), and the reverse side (c), and the nanosheets collected on the membrane filter after dispersion in aq. solution (d) and the magnified image (e).

C. *Hetero-Surface Modification of the Nanosheets*

Before casting the PVA film on the nanosheets-adsorbed substrate, a DMSO solution of α-(3-[3-maleimido-1-oxopropyl]amino)propyl-ω-succinimidyl carboxypentyloxy poly(ethylene glycol) (MAL-PEG-NHS, 10 mM) to the substrate for modification of the obverse surface with MAL-PEG. After washing with distilled water, the PVA solution was cast on the substrate and the dried PAA film was peeled off. For modification of α-methoxy-ω-succinimidyl carboxypentyloxy poly (ethylene glycol) (mPEG-NHS) to the reverse side of the nanosheet, conditions under which the PVA film would be insoluble were used: pH 4.0 and saturated sodium chloride. The mPEG-NHS in DMSO (10 mM) was added to the reverse side of the PVA film which was then dissolved in a phosphate buffer at pH 7.0, to allow collection of the nanosheets. Finally, the nanosheets were reacted with H12-SH to modify the obverse side with H12. Fig. 4.(a) shows the freeze-dried sample of the rectangular nanosheets (5 x 10 μm) dispersed in a phosphate buffer. Interestingly, the obverse side was convex due to the surface roughness. The obverse side was modified with H12 and the reverse side with PEG chains. After mixed with activated platelets, the platelets were selectively adhered on the obverse side only as shown in Fig. 4.(b) because this side was modified with H12.

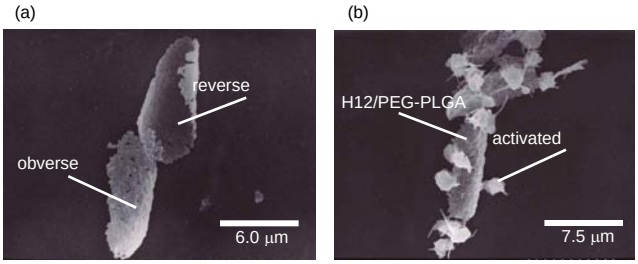

Fig. 4. SEM images of H12-PLGA nanosheets only (a), and H12-PLGA nanosheets interacting with activated platelets.

III. NANOSHEETS WITH SQUERE CENTIMETER BY A LAYER-BY-LAYER METHOD

A. *Free-Standing Giant Nanosheets*

There have been several progressive reports about methodology for the fabrication of giant nanosheets with a huge size-aspect ratio [15, 25-29]. Convenient fabrication or manipulation of nano-scale materials involving a wide variety of macromolecules will significantly enhance the potential applicability of nanotechnology. One novel methodology is a layer-by-layer (LbL) technique [30]. This method is alternative adsorption of oppositely charged polyelectrolytes by electrostatic interaction in accompanied with hydrogen-bonding or hydrophobic interaction [30-34]. LbL-based materials have been applied in several fields such as electrochemical devices, chemical sensors, nano-mechanical sensors, nano-scale chemical/biological reactors and as a drug delivery system [35]. The LbL method has also been used to fabricate a free-standing ultra-thin membrane using a spin-coating assisted LbL (SA-LbL) method [36-38]. We prepare ultra-thin membranes by spin-coating each polyelectrolyte alternatively on a substrate covered with a sacrificial layer (Fig. 5). Subsequent dissolution of the sacrificial layer releases the free-standing ultra-thin membrane within several minutes, unlike conventional protocols that require many hours (e.g., dipping LbL process).

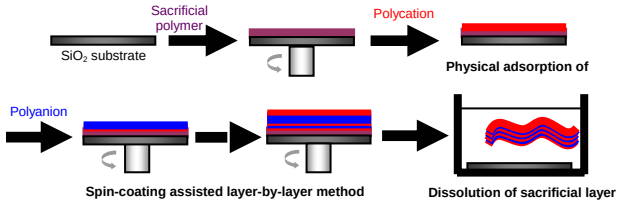

Fig. 5. Preparative scheme of free-standing nanosheets exploiting SA-LbL method.

The free-standing ultra-thin membranes referred to 'polymer nanosheets' of nanometer thickness and a huge aspect ratio ($\geq 10^6$ depending on the size of the substrate) were composed not only by the SA-LbL method but also by a Langmuir-Blodgett method crosslinking amphiphilic copolymers or by a sol/gel method with organic/inorganic interpenetrating networks [26, 27]. These polymer nanosheets have been reported to be well-organized, compliant and robust materials for micro/nano-mechanical studies [39-41]. Herein, we are exploiting the SA-LbL method for the construction of biocompatible giant nanosheets for obtaining the materials without using chemical cross-linking methods but macromolecular assemblies such as electrostatic interaction, which is quite suitable for biomedical application of the nanosheets.

B. *Free-Standing Polysaccharide Nanosheets*

In order to fabricate the nanosheet with the SA-LbL method, we used chitosan and sodium alginate (Na alginate), which have amino and carboxylic groups as cationic and anionic polyelectrolytes, respectively, at ambient pH. These polysaccharides are used in biomedical fields such as wound dressing and artificial skin because of their high biocompatibility and biodegradability.

For the morphological study of the polysaccharide nanosheet surface, the free-standing nanosheet floating in acetone was transferred onto a fresh silicon wafer and observed by AFM. Large-scale (90 μm x 90 μm) topographic images revealed that the nanosheet surface was as smooth and flat as the silicon wafer surface without any corrugations and wrinkles. These results were obtained due to the high-speed horizontal diffusion of the polymers during spin-coating. From the cross-sectional analysis of the nanosheet edge, the thickness of the nanosheet with 10.5 layer pairs of polysaccharides was 30.2 $\pm$ 4.3 nm corresponding to the ellipsometric thickness of the nanosheet directly assembled on the SiO_2 substrate [42]. Of course, the thickness of the nanosheet can be increased by increasing the number of polysaccharides pairs. Interestingly, the adsorbed nanosheets on the SiO_2 substrate showed a brilliant color change depending on the adsorbed number of the nanosheet, which is explained in terms of structural color. From this color, we can estimate the thickness and smoothness of the nanosheet.

C. Nano-Adhesive Plaster: Ubiquitous Transference of the Nanosheets [42]

The free-standing giant nanosheet (PLA nanosheet; 4 x 4 cm with 20-nm thick) floating in water (Fig. 6.(a)) can be scooped with a wire loop as shown in Fig. 6.(b). We surprised that the nanosheet with the loop was stable more than 6 months in air. In order to transfer the nanosheets from the surface of one substrate to another without distorting the overall shape, we used a water-soluble sacrificial membrane such as PVA membrane as a substrate of the polysaccharide nanosheet or the sacrificial membrane plus a supporting film such as silicon rubber for handling. Here, we named this layered composite film a 'nanoadhesive plaster' because we envisaged the nanosheet could attach to skin or organ and water-soluble membrane as the substrate of the nanosheets was finally dissolved.

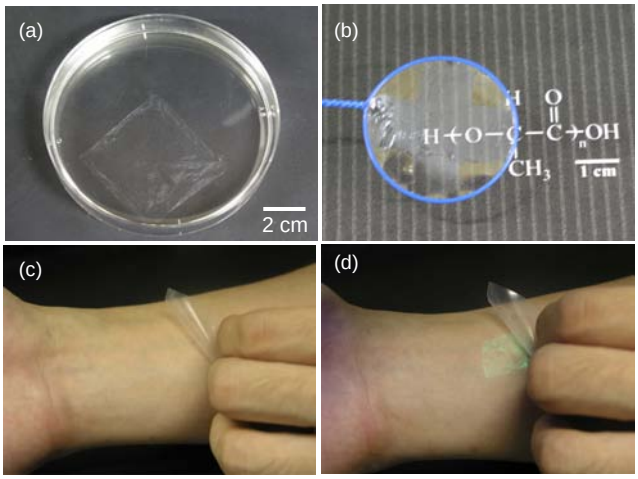

Fig. 6. A free-standing polysaccharide nanosheet floating in water (a), the nanosheet scooped with a wire loop (b), A nanoadhesive plaster on the human skin (c) after release from the silicone rubber. (d) is the same image as (c) except that it was captured in the dark.

We chose PVA as a suitable material for the sacrificial membrane because it is a water-soluble polymer that does not adversely affect the living body. With the PVA membrane, the polysaccharide nanosheet, modified with a small amount of commercialized luminescent pigment for ease of visibility in the dark, was scooped onto the PVA membrane on the SiO_2 substrate or a silicon rubber.

To test the practical applications of the nano-adhesive plaster, we attached it to the skin on the right arm of a human subject. The polysaccharide nanosheet was then released from the PVA-silicone rubber substrate within a few seconds by the dissolution of the PVA layer with a few drops of pure water through a micropipette. The nanosheet on the skin was barely visible from the top view under visible light (Fig. 6.(c)). Luminescent signals from the modified nanosheet confirmed that the shape and size of the polysaccharide nanosheet were preserved on the skin. Furthermore, luminescent regions were barely detected on the removed silicone rubber side, demonstrating the successful transference of the nanosheet onto the skin (Fig. 6.(d)). Moreover, the configuration of the polysaccharide nanosheet was stable for at least 24 h, despite perspiration from the skin and the possibility of it being rinsed away by washing with soap. The polysaccharide nanosheet was no longer visible on the skin, perhaps because the surface relief

As another example, we attached the nano-adhesive plaster to the cecum of a living rat. The adhesion of the polysaccharide nanosheet supported by water-soluble PVA membrane was observed from the luminescent signals (Fig. 7.(a)). Then the PVA membrane was dissolved with a few mL drop of saline through the syringe. Luminescent signals from the modified nanosheet confirmed that the shape and size of the polysaccharide nanosheet were preserved on the cecum. Furthermore, overall shape of the polysaccharide nanosheet was completely fitted on the surface relief of the organ due to high flexibility of the nanosheet and occurrence of the firm tissue-adhesion due to polysaccharide nanosheet was not observed (Fig. 7.(b)). These results indicated that the polysaccharide nanosheet was successfully transferred onto the tissue surface by dissolution of the PVA membrane and quite stable on the tissue surface as little as inducing immunological response. We would prospect the potential biomedical application of the free-standing polymer nanosheet as unique surgical or cosmetic materials.

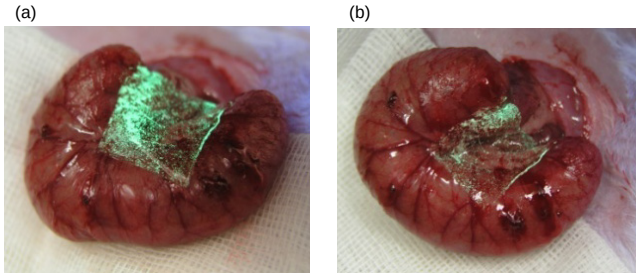

Fig. 7. Nano-adhesive plaster on rat cecum: (a) before and (b) after dissolution of supported PVA membrane.

IV. Conclusions

We succeeded in the preparation of two types of free-standing polymeric nanosheet envisaging biomedical application. The one is an injectable nanosheet as a platelet substitute or an antiplatelet material, which was constructed on the micro-patterned substrate. The other is a giant nanosheet constructed by layer-by-layer assembly with huge size-aspect ratio over 10^6, customized to nanoadhesive plasters which ubiquitously transferred the nanosheet onto human skin or tissues. Now, we have opened new gerontic applications such as hemostasis, thrombosis, sclerosis, wound dressing, skin care, cosmetics, etc.

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