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Fabrication of Bio-friendly Polymer Nanosheets for Biomedical Applications

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We have developed self-standing bio-friendly nanosheets for biomedical applications. These nanosheets were fabricated by simple processes of spin-coating and a novel peeling technique from the substrate. Centimeter-sized nanosheets have unique properties such as amazingly flexibility and high adhesiveness. They could act as a novel wound dressing instead of conventional suturing in surgery. By contrast, fragmented submillimeter-sized nanosheets could act as aqueous surface modifiers to coat even uneven and irregular surfaces in addition to flat surfaces. In fact, such fragmented nanosheets exhibited a high potential to protect burned skin from bacterial infection and to provide blood compatibility to several surfaces. These nanobiomaterials therefore constitute a promising alternative to conventional therapies and coatings in biomedical fields.
Key words: *Nanosheet, Bio-friendly polymer, Wound dressing, Aqueous surface modifier, Biomedical application*

1. INTRODUCTION

In nanotechnology research, much attention has been focused on the preparation of self-standing ultra-thin films, which are frequently called nanosheets and nanomembranes. The nanosheets represent unique properties such as high transparency and flexibility due to their dimensions (centimeter size but only tens-of-nanometers thickness) [1]. The techniques that have been adopted to prepare these sheets include the utilization of polymers and/or inorganic materials such as layer-by-layer nanosheets of polyelectrolytes [2-4], assemblies of triblock copolymers [5], cross-linked self-assembled monolayers [6], amphiphilic Langmuir-Blodgett nanosheets [7,8], sol/gel interpenetrating network nanomembranes [9], and graphene nanosheets dissociated from graphite [10]. These nanosheets have been developed for various research fields, e.g. applications in electrochemistry and separation technology such as nanosensors, energy storage, and selective adsorptions [1].

For biomedical applications of the nanosheets, we have focused on two types of bio-friendly polymers. One is typical biodegradable polyester series such as poly(lactic acid) and their copolymers. Such polyesters have been clinically applied as biodegradable sutures [11] and bone screws [12] etc. and also have been widely explored as injectable carriers for drug delivery [13]. Therefore, utilization of biodegradable polyesters would be the shortest route to clinical application of nanosheets. The other type is biocompatible polymer series containing a phosphorylcholine (PC) group, which is one of the polar components of phospholipids in cell membranes [14]. A representative example is a 2-methacryloyloxyethyl phosphorylcholine polymer synthesized by Ishihara *et al.* that exhibits good blood compatibility and that efficiently reduced the nonspecific adhesion of proteins and cells to the polymer surface [15,16]. We have recently succeeded in synthesizing diamine and diol aromatic monomers containing the PC group to provide thermally stable and mechanically robust properties [17,18]. Using these monomers, we can synthesize various aromatic polymers by polycondensation or polyaddition of polyamides, polyimides, poly(urethane-urea)s, polyesters and polyurethanes [19-22]. In fact, these polymers exhibited good mechanical properties, in addition to good biocompatibility [20].

In this review, we introduce simple preparative methods for generating self-standing nanosheets composed of bio-friendly polymers as described above, which would be required for biomedical applications. Moreover, we introduce biomedical applications of these size-controlled nanosheets, e.g. innovative wound dressings for incised organs and burned skin, and aqueous surface modifiers to provide biocompatible surfaces. We focus on these aspects because abundant excellent reviews on the self-standing nanosheets and their applications have been published previously [1,23].

2. PREPARATION OF SELF-STANDING NANOSHEETS

2.1 Centimeter-sized Nanosheets

Self-standing centimeter-sized nanosheets can be obtained from substrates by a “sacrificial layer method” or a “supporting layer method” (**Fig. 1a**) [24,25]. In the former case, the sacrificial layer is dissolved with solvents that do not dissolve the nanosheets. We present poly(vinyl alcohol) (PVA) as an example of a water-soluble sacrificial layer to obtain self-standing nanosheets composed of poly(L-lactic acid) (PLLA) [25]. First, an aqueous solution of 10 mg/mL PVA was pipetted onto a silicon wafer (SiO_2 substrate) cut to an appropriate size. The substrate was spin-coated for 20 s at 4,000 rpm, followed by drying at 70°C. The PVA-coated substrate was next spin-coated with a 5 mg/mL of PLLA solution under the same conditions.

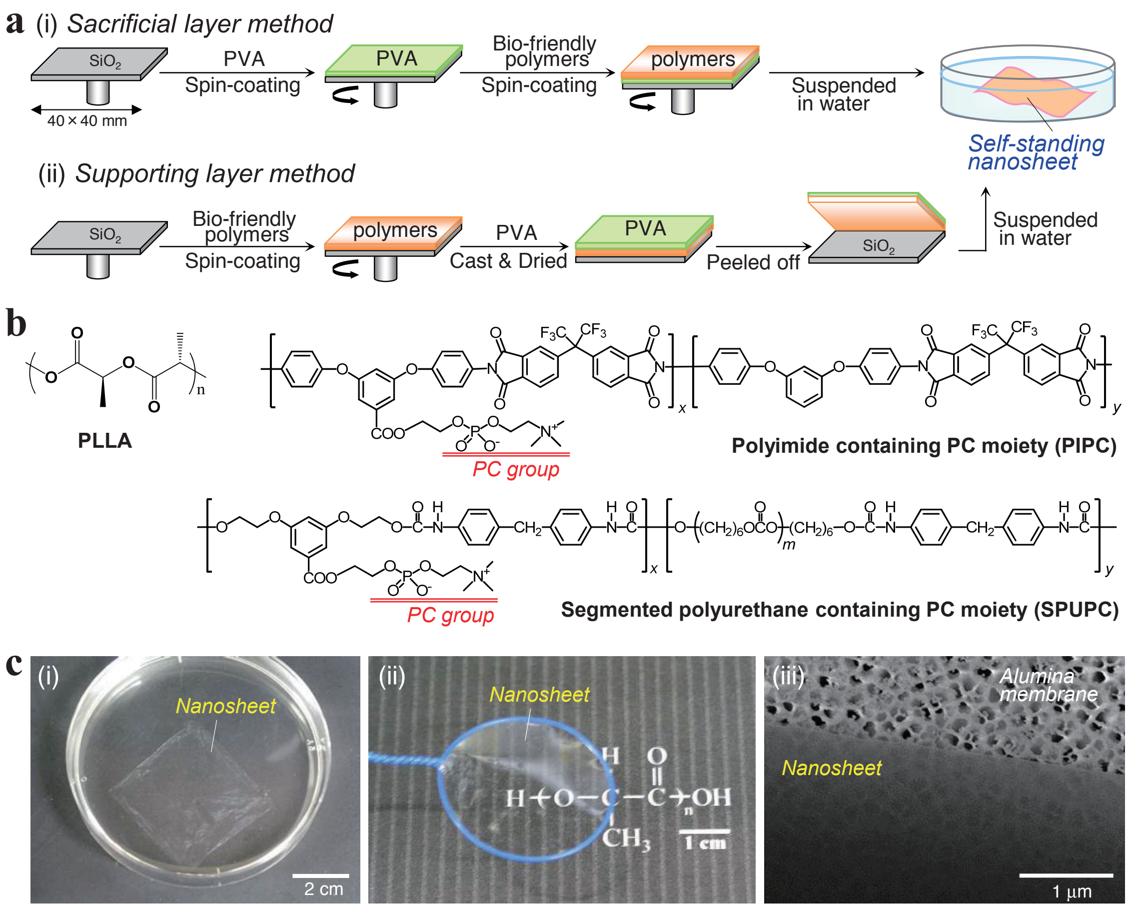

Fig. 1 Self-standing bio-friendly centimeter-sized polymer nanosheets. **(a)** Preparative scheme of the self-standing nanosheet by two kinds of the simple processes: (i) sacrificial layer method or (ii) supporting layer method. **(b)** Chemical structures of bio-friendly polymers used in this review. **(c)** The obtained PLLA nanosheets (i) suspended in water, (ii) scooped up with a wire loop, and (iii) SEM image of a PLLA nanosheet adhered to an alumina membrane. (Cited and partially modified from ref. 26)

When the substrate was immersed into water, the PLLA nanosheets were instantly detached from the substrate by dissolving only the PVA sacrificial layer with water. The thickness of the nanosheets thus became approximately 20 nm and the roughness was as low as several nanometers. Moreover, we confirmed that the thickness was proportional to the concentration of PLLA utilized for spin-coating. Using this technique, we can prepare nanosheets composed of versatile polymers (poly(styrene) and poly(vinyl acetate) etc.), typical biodegradable polymers (poly(lactide-*co*-glycolide) and poly(caprolactone), etc.) and other bio-friendly polymers (polyimide and segmented polyurethane containing the PC moiety, **Fig. 1b**).

In the case of "supporting layer method", we have proposed a novel technique to peel nanosheets from the substrates by using a water-soluble supporting layer such as PVA [26]. This coating procedure is the other way around compared to the sacrificial layer method. First, a 5 mg/mL solution of PLLA was directly spin-coated on the substrate at 4,000 rpm for 20 s. We confirmed that the surface of the substrate became slightly red in color. Subsequently, a 100 mg/mL solution of PVA was cast on the surface of the PLLA-coated substrate and then dried at 70°C. When the dried PVA layer was detached from the substrate with tweezers, the light red color of the substrate disappeared, indicating that the PLLA nanosheet had been transferred to the PVA layer. In fact, we could obtain a self-standing PLLA nanosheet with a thickness of approximately 20 nm by dissolving the PVA layer with water (**Fig. 1c (i)**) [26]. This could be the reason why the van der waals interaction and hydrogen bonding between the PVA and the PLLA nanosheet could be greater than the interaction of the dried nanosheet with the SiO_2. It is notable that this transfer technique by the supporting layer is an efficient means of improving the handling of the nanosheet and attaching the nanosheet from the substrate to other surfaces including skin and organs, without utilizing any organic solvents. The resulting nanosheets, which had a high size-aspect ratio more than 10^6, were transparent, flexible, and they maintained the size of the SiO_2 substrate (**Fig. 1c (ii)**) [26]. SEM observation of the nanosheets scooped on an alumina membrane confirmed that the nanosheet had a flat surface without cracks (**Fig. 1c (iii)**) [26].

2.2 Characterization of centimeter-sized nanosheets

The mechanical properties of the PLLA nanosheets were measured using a bulging test developed for thin films [27]. The nanosheet with a thickness of ca. 20 nm was physically adhered to a steel plate with a hole of 1 mm diameter. The plate was fixed on a custom-made chamber and pressure applied to the nanosheet and its deflection over time was monitored. Based on the slope of the elastic region of the stress-strain curve, the Young's modulus of the nanosheet was calculated to be 1.7 ± 0.1 GPa [26]. This value was significantly lower than that of the bulk PLLA film (7-10 GPa) as reported previously [28]. This means that the PLLA nanosheet was softer than the bulk PLLA. Mattsson *et al.* have reported that the glass transition temperature (T_g) of poly(styrene) films with a thickness of 22 nm was obviously decreased to 37°C, as compared to bulk poly(styrene) (T_g: 109°C) [29]. This result may be one of the reasons why the T_g of the PLLA nanosheet would be lower than that of bulk PLLA (T_g: 60°C), as the interactions between polymer chains decreased in the thin films [29]. Unfortunately, the Young's moduli of the nanosheet with thickness more than 200 nm could not be analyzed with the bulging test due to detaching of the nanosheet from the steel plate, resulting in leakage. However, this was important evidence that the thickness of the PLLA nanosheet should be related to its adhesiveness.

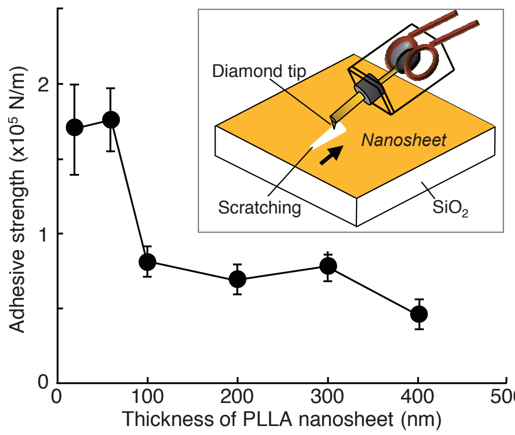

Fig. 2 Adhesive strength of the PLLA nanosheets with different thicknesses adhered on the SiO_2 substrate. Inset shows a schematic image of the scratch tester for thin films. (Cited and partially modified from ref. 26)

Therefore, we explored the correlation between adhesive strength of the nanosheets and their thickness using a scratch tester for thin films [30], where the diamond tip horizontally scratched the PLLA nanosheets adhered on the SiO_2 substrate (**Fig. 2 inset**). The critical load just after detaching the nanosheet with a thickness of ca. 20 nm was calculated to be 1.7 ± 0.3 × 10^5 N/m, which was equivalent that of a nanosheet with a thickness of ca. 60 nm (**Fig. 2**) [26]. However, the critical load of the nanosheets over 100 nm thickness was significantly decreased. This indicates that the nanosheets could conform to the roughness of the desired surfaces due to its great flexibility and low roughness. In fact, the nanosheets have a unique potential to adhere to various surfaces such as glasses, steels, moist skin and organs, without the use of adhesive reagents. Once the nanosheet had dried on the surface, it was difficult to detach by scratching with tweezers. We thus revealed that the greatest benefit of the nano-thickness of PLLA is its high potential to adhere.

2.3 Fragmented submillimeter-sized nanosheets

As described above, we have proposed the use of self-standing centimeter-sized nanosheets, which exhibit interesting properties such as high adhesiveness, flexibility and high transparency. However, such nanosheets are only suitable for sealing of relatively flat and broad surfaces due to the dimensions of the nanosheets. They are often hard to attach to irregular and uneven surfaces, however. In this section, we introduce fragmented submillimeter-sized nanosheets to effectively coat irregular and uneven surfaces, in addition to flat and broad surfaces.

We succeeded in preparing abundant self-standing centimeter-sized PLLA nanosheets [31]. According to **Fig. 3a**, a 100 mg/mL solution of PVA as a water-soluble sacrificial layer was dropped on a SiO_2 substrate, followed by a spin-coating at 4000 rpm for 20 s. Next, a 10 mg/mL solution of PLLA was spin-coated on the PVA-coated substrate under the same conditions. The surface of the substrate was slightly red in color, and its thickness was approximately 60 nm. Next, the multi-layering of PVA and PLLA was repeated dozens of times on the SiO_2 substrate. By dissolution of PVA layers in water, we could obtain many PLLA nanosheets, corresponding to the number of multi-layering processes of PVA and PLLA. Therefore, we succeeded in dramatically improving the preparation of self-standing PLLA nanosheets by a simple multi-layering process combined with a peeling technique.

We have proposed that the centimeter-sized PLLA nanosheets can be easily fragmented with a homogenizer. The surface area of one nanosheet before homogenization was approximately 1,600 mm^2, corresponding the size of the SiO_2 substrate (40 × 40 mm). When the nanosheets were homogenized at 30,000 rpm, they were quickly fragmented, and the surface area of one fragmented nanosheet reached a minimum at 0.24 ± 0.08 mm^2 within 10 min [31]. Interestingly, the fragmented nanosheets were homogeneously suspended in water, where the turbidity and viscosity of the suspension were significantly increased (**Fig. 3b**). Using this technique, we could prepare fragmented nanosheets composed of other bio-friendly polymers: PC-containing polyimide (PIPC) and segmented polyurethane (SPUPC).

2.4 Characterization of fragmented submillimeter-sized nanosheets

We tested the behavior of the coating with the fragmented PLLA nanosheets, which were just cast and dried on the bare SiO_2 substrate. Interestingly, the fragmented nanosheets were adhered to the substrate in a spread out configuration (no aggregation) (**Fig. 3c**) [31]. The coating behavior of the nanosheets looks like "patchwork", based on the colors of the nanosheets adhered on the substrate (**Fig. 3c**) [31]. Once the nanosheets had dried onto the surface, they were difficult to detach by scratching with tweezers. These results indicate that the nanosheets are solidly adhered to each other via a surface-contact interaction. Next, we investigated whether the irregular and uneven surfaces

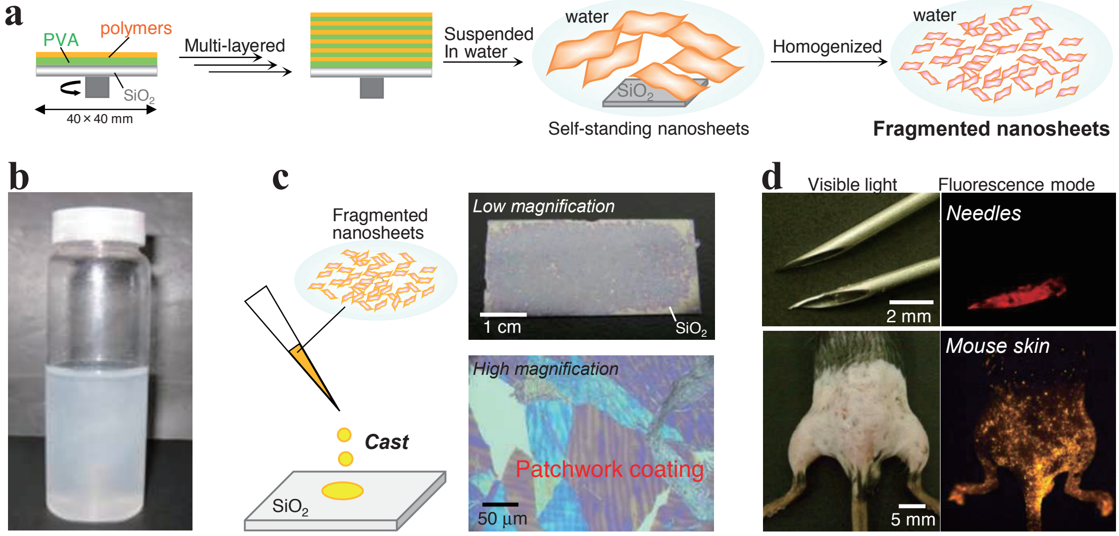

Fig. 3 Fragmented submillimeter-sized nanosheets. **(a)** Preparative scheme of the fragmented nanosheets. **(b)** Macroscopic image of the fragmented PLLA nanosheets suspended in water. **(c)** Schematic (Left) and macroscopic (Right) images of patchwork coating with the fragmented PLLA nanosheets on a SiO_2 substrate. **(d)** Patchwork coating of the nanosheets on the needles and on the lower part of the mouse body. (Cited and partially modified from ref. 31)

are effectively coated with the patchwork coating of the fragmented nanosheets. To this end, we prepared a suspension of fluorescent-labeled nanosheets to visualize their coating behavior. When several surfaces such as a needle and the lower part of the mouse body, which has an irregular shape, were immersed into the suspension, they were effectively coated with the nanosheets (**Fig. 3d**) [31]. On the other hand, the coatings were hardly detectable under visible light. This indicates that the ultra-thin nanosheets could be adhered along the roughness of the desired surfaces at the nanometer scale. This is a unique property of the fragmented nanosheets generated by the patchwork coating method.

3. BIOMEDICAL APPLICATIONS OF NANOSHEETS

3.1 Centimeter-sized Nanosheets for sealing operations

Tissue repair after surgery has classically been performed by suture and ligation, which are highly reliable operations. However, tissue repair by suture and ligation may occasionally become difficult and unreliable when the repaired tissue is fragile and edematous. In such cases, the likelihood of anastomotic breakdown is increased. Especially in case of gastrointestinal surgery, severe sepsis is considered to be one of the most critical complications. Therefore, an improved operation technique to effect tissue repair is required to replace suture and ligation. As described in the Introduction, biodegradable polyesters have been clinically applied as biodegradable sutures [11]. We have demonstrated a potential biomedical application of the centimeter-sized PLLA nanosheets as a wound dressing to repair gastric incisions by sealing, instead of suture.

The sealing ability of the PLLA nanosheets was evaluated *in vivo* using gastrotomy model mice. The stomach wall was incised with a surgical knife. Afterwards, the nanosheets were transferred to a PVA fi-

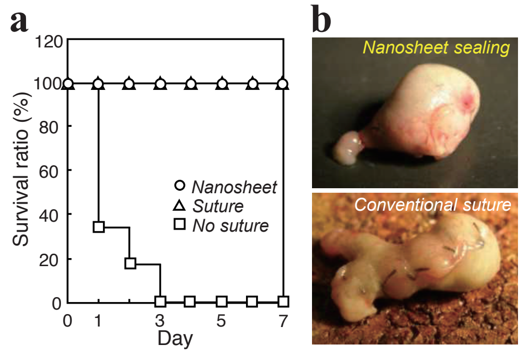

Fig. 4 Sealing effect of the centimeter-sized PLLA nanosheet in gastrotomy model mice. **(a)** Survival studies of mice treated with (○) nanosheet, (△) classical suture surgery, or (□) non-suture as a negative control (n=7-10). **(b)** Macroscopic images of stomachs treated with nanosheet (upper image) or conventional suture (lower image) after 7 days. (Cited and partially modified from ref. 26)

lm prepared by the supporting layer method (**Fig. 1a**) and sealed to the incised site. The PVA film was dissolved with saline before abdominal closure and the mortality of the mice over the following week was recorded. The incised site was sufficiently sutured or not sutured as positive and negative controls. All of the non-sutured mice died of massive leakage after 3 days, whereas all classically suture-treated mice survived (**Fig. 4a**) [26]. Interestingly, all of the mice treated by the nanosheet-sealing without any suture method survived. Removal of their stomachs 7 days after surgery revealed that the nanosheet did not cause tissue adhesion or post-operative scars on the stomach (**Fig. 4b**) [26]. In contrast, the sutured mice showed stomach deformation, tissue adhesion and apparent scarring (**Fig. 4c**) [26]. These results indicate that the nanosheet could act as a biointerface to balance the conflicting phenomena involved in tissue repair and resistance to tissue adhesion. These results demonstrate that the PLLA nanosheets could act as a novel, effective wound dressing as an alternative to conventional suturing operations.

3.2 Fragmented nanosheets as patchwork treatment for burn wounds

A burn wound is a skin or flesh wound caused by heat, chemicals, electricity etc. Much attention is paid to minimize the risk of infection during wound healing in the control of burn wounds [32]. Otherwise, superficial and partial thickness burn wounds could evolve to deeper tissue damage. One of the most crucial complications is severe sepsis resulting from infection. Different types of wound dressings have been applied for the treatment of the burn wounds [33]. Though these dressings are suitable for wrapping flat interfaces such as the belly and back, it is often difficult to wrap burn wounds having an irregular or uneven shape, such as fingers and between fingers, etc. Herein, we focused on the unique properties of fragmented nanosheets generated using the "patchwork coating technique". We tested whether patchwork coating protects the irregular and uneven burn wounds from the infection in a mouse model system [31].

In order to provide a patchwork coating, fragmented nanosheets composed of PLLA were dropped and dried onto the region of the burn wound. Next, *Pseudomonas aeruginosa* bacteria was added to the patchwork region. Skins were then obtained from the mice after 3 days and stained with Hematoxylin-Eosin for histological analysis. In the samples treated with a patchwork coating, the bacteria stained blue-purple remained on the dermis and did not migrate into the dermis of the dorsal skin (**Fig. 5a**) [31]. In the negative control without patchwork, the bacteria migrated into both the dermis and the subcutaneous layer. This means that the burn wound was infected with the bacteria (**Fig. 5b**) [31]. We thus demonstrated that patchwork coating could act as a novel barrier for burn wound treatment to inhibit the infection. Thus patchwork coating may constitute a promising alternative to conventional therapy for burn patients.

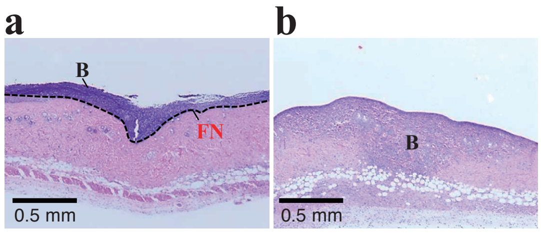

Fig. 5 *In vivo* barrier effect of patchwork coating of the fragmented PLLA nanosheets against burn wounds. **(a)** Histological image of skin with patchwork coating. The bacteria (*Pseudomonas aeruginosa*) remained on the patchwork coating as depicted by a dotted line. **(b)** Negative control (without patchwork). The bacteria infiltrated both the dermis and subcutaneous layer. Letters B and FN indicate bacteria and fragmented nanosheets, respectively. (Cited and partially modified from ref. 31)

3.3 Fragmented nanosheets as aqueous surface modifiers to provide blood compatibility

As described above, patchwork coating acted as a barrier to protect burn wounds from the infection. In other words, this means that the patchwork coating changed the surface property of infectible burn wounds. We were inspired from these results to explore the application of patchwork coating as an aqueous surface modifying technique. Platelets are blood cells involved in normal hemostasis as well as pathological thrombosis. A critical point in the application of blood compatible materials is to inhibit interactions between platelets and the surface of the materials, to avoid pathological thrombosis. We focused on the fragmented nanosheets composed of the polyimide containing PC moiety (PIPC) to provide blood compatibility to several surfaces.

Using the same methodology described in section 2.3, we prepared fragmented PIPC nanosheets with a PC unit of 14 mol%, which were homogeneously suspended in water (**Fig. 6a**). The thickness of each nanosheet and the surface area of one fragmented nanosheet were 20 ± 4 nm and 6800 ± 208 μm^2, respectively. When the fragmented nanosheets were cast and dried on a bared poly(ethylene terephthalate) (PET) plate as a model surface, they consisted of a patchwork, similar to the fragmented PLLA nanosheets (**Fig. 3c**).

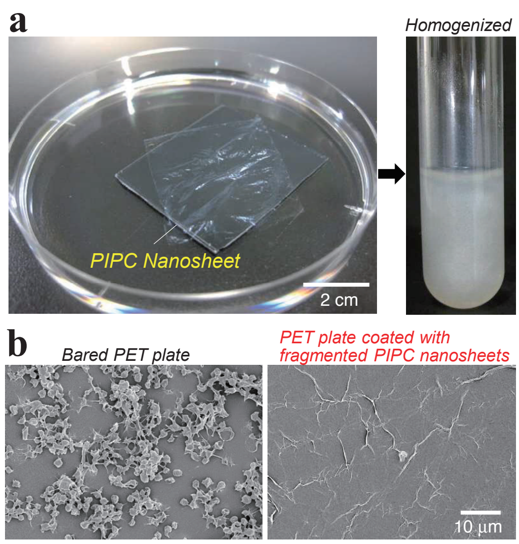

Fig. 6 Patchwork coating of the fragmented PIPC nanosheets with excellent blood compatibility. **(a)** Macroscopic images of the PIPC nanosheets before and after homogenization. **(b)** Platelet adhesion test. SEM images of the PET plate with (right) and without (left) patchwork coating with fragmented PIPC nanosheets, to which human platelet-rich plasma was added and incubated at 37°C for 2 h.

Next, we tested the blood compatibility of the PET plates coated with the fragmented PIPC-nanosheets. To this end, human platelet-rich plasma was applied to the plates, followed by an incubation at 37°C for 2h. As depicted in **Fig. 6b**, no platelets adhered to the plate. Moreover, we observed some white lines on the plates, which corresponded to wrinkles (not cracks) formed during the patchwork process. This indicated that they were solidly adhered to each other in a spread out conformation. In the case of the bared PET plates, abundant platelets with filopodial extensions were non-specifically adhered to the plates (**Fig. 6b**). These studies revealed that patchwork coating with the fragmented PIPC nanosheets acts as an aqueous surface modifier to provide blood compatibility.

5. SUMMARY

For biomedical applications, we have developed two types of self-standing bio-friendly nanosheets using simple fabrication processes consisting of a spin-coating and a novel peeling technique. The first application, centimeter-sized nanosheets, constitutes a promising alternative to conventional suture operations in surgery, e.g. to create innovative wound dressings for incised organs. The second application, fragmented submillimeter-sized nanosheets, acted as aqueous surface modifiers to effectively coat even irregular and uneven surfaces. Such a coating technique has the potential to protect burned skin from the bacterial infection and to provide blood compatibility to various surfaces. We expect that these kinds of nanosheets, with their unique properties, may possess high potential as new nanomaterials with applications in various research fields, e.g. medicine, energy and environmental sciences.

6. ACKNOWLEDGEMENTS

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