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Materials Letters 72 (2012) 88–91

Contents lists available at SciVerse ScienceDirect

Materials Letters

journal homepage: www.elsevier.com/locate/matlet

Hydrophilic-modified polyurethane nanofibre scaffolds for culture of hyperthermophiles

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ARTICLE INFO

Article history:
Received 15 September 2011
Accepted 27 December 2011
Available online 02 January 2012

Keywords:
Electrospinning
Nanofibre
Hyperthermophiles
Attachment
Scaffold

ABSTRACT

Hyperthermophiles are expected as the source of thermostable enzymes, but agarose-gel culture is unavailable for isolating them because of the high temperature culture condition. In this paper, we proposed the scaffold suitable for long-term culture of hyperthermophiles, which is applicable for an extremely high-temperature. We have investigated the dynamics of the attachment and colonisation of *Sulfolobus solfataricus*, onto electrospun nanofiber scaffold. Observation by fluorescent microscopy and SEM demonstrated adhered and colonised onto the polyurethane nanofibres the hydrophilicity of which was enhanced by oxygen plasma treatment. This research is a first step towards developing a new approach to successful solid-culture of hyperthermophiles under extreme conditions.

1. Introduction

Hyperthermophiles, isolated from continental and marine volcanic environments, grow at near 100 °C [1]. They have different metabolic pathways and enzymes from those of bacteria and eukarya [2,3] that they are expected as the source of novel enzymes stable in extreme conditions; high temperature, high pH, low pH, and high concentration of organic solvent. Therefore, they are not only biological concerns for the advancement of research, but also the concern with their industrial application has been growing [4].

To isolate and culture hyperthermophiles, the liquid medium has been conventionally employed instead of the solid agarose-gel medium for common bacteria and other cell types [4–6]. Liquid medium burdens the isolation of colony because picking up a single colony from liquid medium is difficult. The main reason why the solid medium has not been used in their culture is due to the long cultivation time. Their proliferation is slow and, therefore, it was difficult to keep the medium volume at high temperature preventing vaporisation. Thus, the improvements are needed in the recovery of these 'unculturable' microorganisms [7,8]. Furthermore, it is difficult to prepare agarose gel at high temperature, which hyperthermophiles favour. To address these problems, the culture scaffold is highly desired instead of solid-gel medium. But, the main hindrance to cultivation is the prevailing lack of knowledge on the initial attachment and colonisation of the scaffold [9–11]. This research is a first step in the culture scaffold of *Sulfolobus solfataricus*, hyperthermophiles living at high temperature and low pH. To prepare the nanofiber scaffolds, we employed electrospinning. This process is widely used in the culture scaffold for mammalian cell and medical devices, [12,13] because it is convenient and inexpensive to fabricate scaffolds, applicable to use various polymers and easy-to-control dimensions of scaffolds.

2. Materials and methods

2.1. Fabrication of nanofiber scaffolds by electrospinning

Polymer solutions chosen were shown in Table 1. Polyurethane (PU; P22SRNAT, JIS hardness; 82A) was purchased from Nippon Polyurethane Industry (Tokyo, Japan), polystyrene (PS; Mw 20,000) from Sigma-Aldrich (MO, USA), tetrahydrofuran (THF) and dimethylformamide (DMF) from Wako Pure Chemical Industries (Osaka, Japan).

The electrospinning set-up (MECC Co., Ltd., Fukuoka, Japan) is detailed in Scheme 1A. A polymer solution is loaded into a syringe and driven through a metallic needle at a constant feed rate by a syringe pump, forming a droplet at the tip of the needle. A high voltage is applied between the tip and the rotating collector grounded. Electrospinning parameters tested are listed in Table 1.

To enhance colonisation nanofibers, each polymer was electrospun over a unique 'bridging' system, lifting the nanofibres from the cover slip, allowing more surface area to be colonised, see Scheme. 1B. Glass slides (cover slip, 22 mm × 26 mm, thickness no. 5; Matsunami, Osaka, Japan), were used as a base, upon which two pieces of conductive double adhesive tape (thickness 0.2 mm) were applied. Silicone rubber

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doi:10.1016/j.matlet.2011.12.103

Table 1
Polymer solutions and the condition of electrospinning.

#	Polymer	Solvent	Conc., w/v%	Voltage, kV	Infusion rate, mL/h	Collector rotation, rpm
1	PU	95% THF/ 5% DMF	15	25	0.8	1200
2	PS	THF	20	25	0.7	900
3	PS	THF	30	25	1.0	900

(thickness 0.3 mm) was then placed on the tape. This arrangement lifted the fibres 0.5 mm up and away from the surface of the glass slide. The optimum distance was 7 mm between lifts. To improve surface hydrophilicity of PU and PS nanofibres, oxygen plasma treatment (100 W, 30 s, 0.1 MPa, chamber size, diameter 64 mm × depth 160 mm) was carried out using a plasma reactor (PR300; Yamato Scientific, Tokyo, Japan).

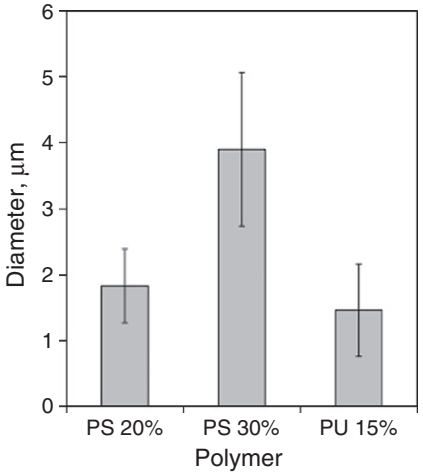


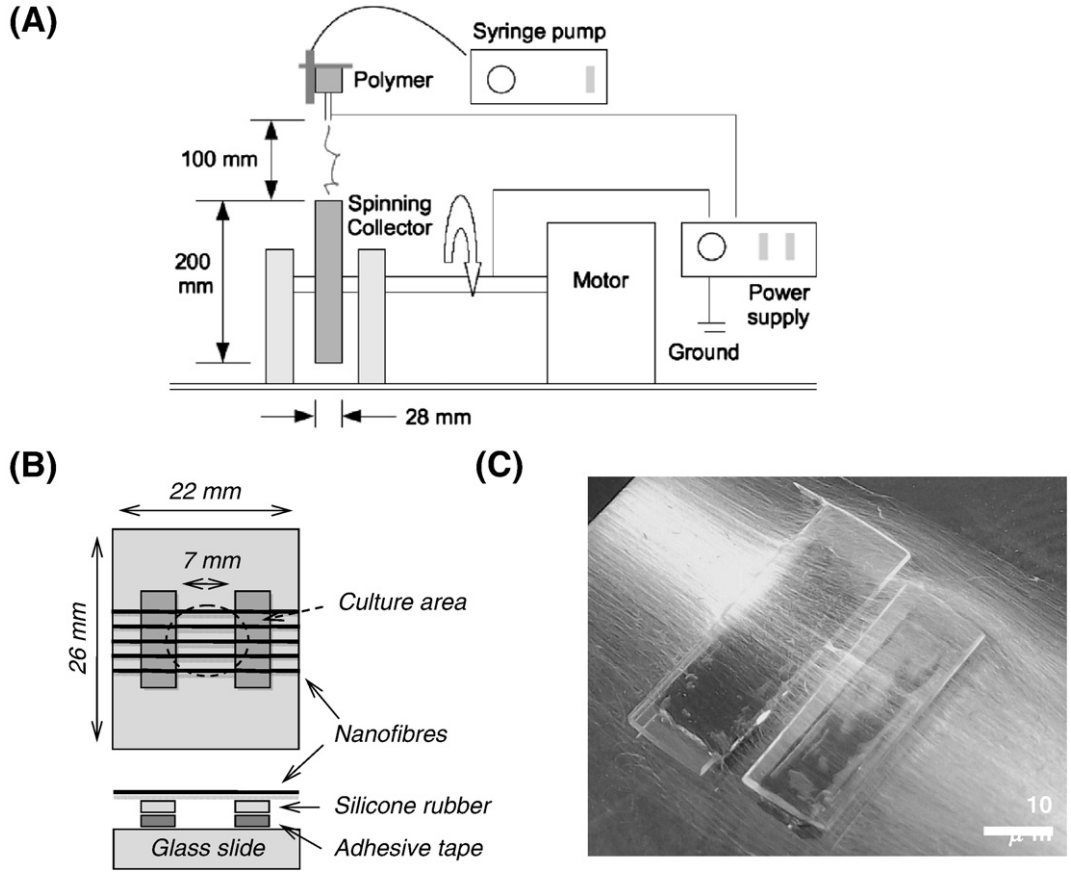
Fig. 1. Diameters of fabricated nanofibres. Means ± SD.

2.2. Culture conditions

The thermoacidophilic archaeon *S. solfataricus* P1 (JCM11322) obtained from Japan Collection of Microorganisms (Saitama, Japan). It was cultured aerobically in medium containing casamino acids (1 g/L), yeast extract (1 g/L) and the following trace minerals; $(NH_4)_2SO_4$ (4.9×10^{-2} M), KH_2PO_4 (2.1×10^{-3} M), $MgSO_4$ (1.0×10^{-3} M), $CaCl_2$ (4.8×10^{-4} M), $FeCl_3$ (7.1×10^{-5} M), $MnCl_2$ (9.1×10^{-6} M), $Na_2B_4O_7$ (1.2×10^{-5} M), $ZnSO_4$ (7.7×10^{-7} M), $CuCl_2$ (2.9×10^{-7} M), Na_2MoO_4 (1.2×10^{-7} M), $VOSO_4$ (1.5×10^{-7} M), $CoSO_4$ (6.5×10^{-8} M), adjusted pH 3.0 with 10 N H_2SO_4 [14]. One millilitre of the seed culture was inoculated onto nanofibres scaffold in a 35-mm polystyrene suspension culture dish (Corning, NY, USA) with 3 mL of medium. The dish was taped firmly and incubated at 80 °C for 1 week.

2.3. Fluorescent microscopy

Cultures were washed twice in phosphate buffered saline (PBS) to remove non-adherent cells then fixed with 4% paraformaldehyde (PFA) for 30 min. After the fixed cells were washed with PBS twice, they were stained with Hoechst 33342 (diluted 1:2000; Dojindo Laboratories, Kumamoto, Japan) for 30 min, being left in the dark,



Scheme 1. Schematic illustration of (A) electrospinning set-up and (B) the 'bridging' system, designed to lift and separate individual nanofibers from the glass. Cells are inoculated and cultured onto the bridged fibres between lifts. (C) Digital photograph of the 'bridging' system. Good alignment of PU nanofibres has bridged the 7-mm gap between the two pieces of silicone rubber. Bar = 10 μm.

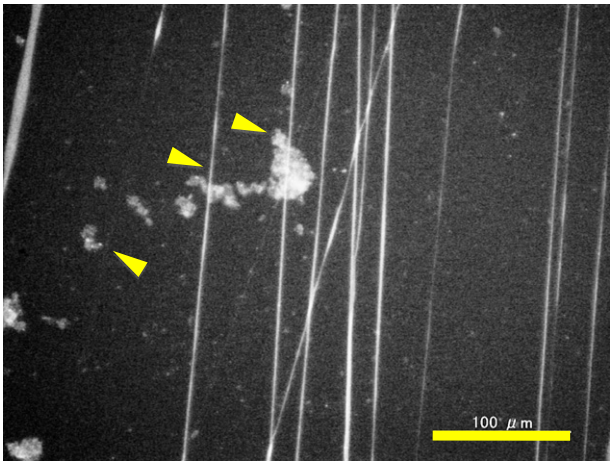


Fig. 2. Fluorescent microscopic observation of *S. solfataricus* which adhered and spread well onto the PU nanofibers after 1-week culture. Arrowheads indicate colonies. Cell nuclei were stained with Hoechst 33342. Fibres showed intrinsic fluorescence. Bar = 100 μm.

and finally washed twice in PBS. The adherent cells on each nanofibre were observed on a fluorescence microscope (BX51; Olympus, Tokyo, Japan).

2.4. Scanning electron microscopy

Cultures were fixed with PFA as described above, dehydrated through a graded series of ethanol (50, 60, 70, 80, 90, 95, 99 and 100%), replaced into 2-methyl-2-propanol (Wako), frozen at 4 °C, and then lyophilised with a vacuum evaporator. Dried samples were sputter-coated with Au/Pt (thickness 20 nm), and observed by a scanning electron microscope (S-2600; Hitachi, Tokyo, Japan) [15].

3. Results and discussion

We focused on the wide application and availability of electrospun nanofibres and applied to the culture scaffold for *S. solfataricus*, hyperthermophiles. In this study, PU and PS were chosen, because they offered many advantages over natural, mainly the ability to produce a wider range of mechanical properties [16]. The fibres uniformly crossing the bridge in a firm alignment were shown in Scheme. 1C. In the preliminary experiment, polylactic-co-glycolic acid (75:25; Mw 20,000; Wako) has been examined, but it has not kept without dissolution for 1 week at culture conditions (80 °C, pH 3.0). In the preparation of PU fibres, DMF was added to the solvent THF to slow the evaporation of the solvent. The concentration of each polymer was determined on the basis of the viscosity. The collector was rotated to align fibres. The collector speed was adjusted for each polymer. The diameters of fabricated fibres were measured by scanning electron microscopy (SEM) and shown in Fig. 1. The diameters of 15% PU, 20% PS and 30% PS were 1.46 ± 0.70, 1.83 ± 0.56 and 3.90 ± 1.17 μm, respectively (mean ± standard deviation). This result shows that PU and PS fibres in the wide range of micron to sub-micron have been made. We have tried to fabricate 12% PU fibre, but it was too thick to make a bridging substrate.

Then, we tried to culture *S. solfataricus* on nanofiber for 1 week. As a result, cell attachment was not observed on PS scaffold, while attachment was observed on PU fibre (Fig. 2). Consistent with the observations by SEM (Fig. 3), *S. solfataricus* attached and colonised onto the 15% PU nanofibers, while they showed no attachment and colonisation onto the same-sized 20% PS fibres. The number of attached cells was 0.95 cells/μm, which was counted by SEM images (the number of counted fibre: n = 57; the total length of counted fibre: 855 μm). We considered that this result is due to the difference of surface chemistry. When the polymer fibres were exposed to the culture medium, proteins in the medium are rapidly adsorbed onto their surface before the cells can adhere. The adsorbed proteins determine the subsequent cell attachment behaviours [17]. In addition, both of the non-plasma treated-PU fibre and non-plasma treated-PS

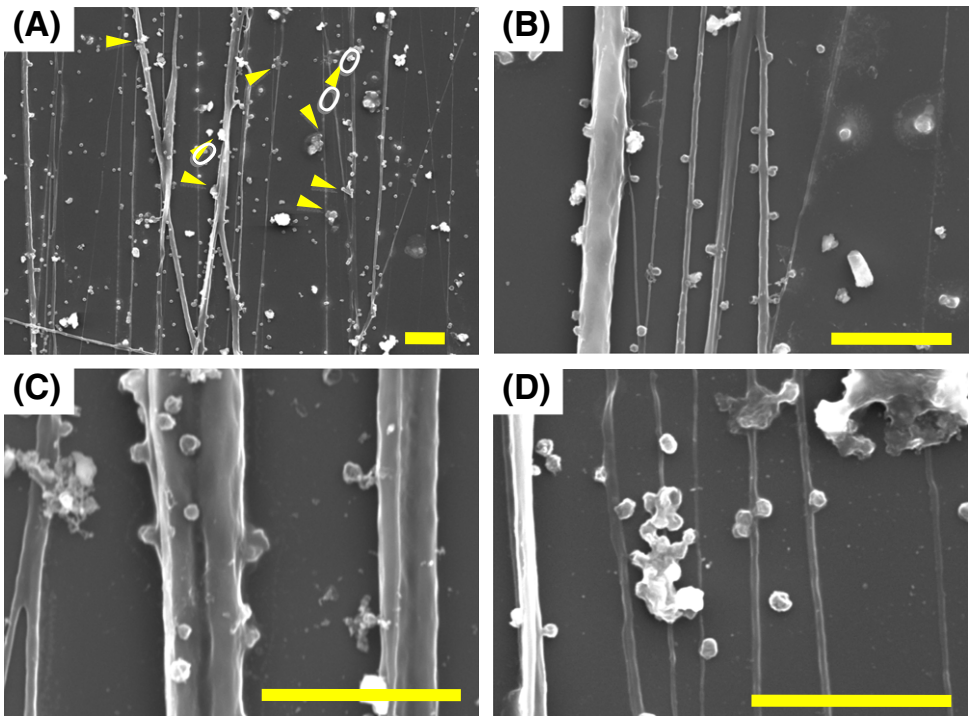


Fig. 3. SEM observation of *S. solfataricus* cultured on PU nanofibers for 1 week. (A) Low magnification image. Arrowheads indicate colonies. (B, C) High magnification images of a single cell attached on a thin (B) or thick (C) fibre. (D) High magnification image of a clump of proliferated cells. Bar = 10 μm.

fibre showed no attachment. It is known that plasmas can be used to alter material surfaces by removing surface layers, to activate the surface hydrophilicity [18]. The water drop contact angles of the cast film of PU and PS by after plasma treatment have been reduced from $94.6° \pm 10.2°$ to $67.9° \pm 1.5°$ and $109.5° \pm 3.9°$ to $20.2° \pm 2.9°$. We considered that the optimal surface for the adsorption of the adhesion protein was moderately hydrophilic surface, as seen in the plasma-treated PU.

4. Conclusions

We have investigated the availability of electrospun scaffold, which showed hydrophilicity by O_2 plasma treatment, and the successful colonisation of hyperthermophiles was observed by SEM. This research is a first step towards developing the scaffolds for cell attachment and colonisation at extreme condition, which leads to the application of extremophiles to environmental materials and industrial usage.

Acknowledgements

This work was supported by the Japanese Society for the Promotion of Science, Summer Program 2011.

References

[1] Adams MW. Enzymes and proteins from organisms that grow near and above 100 °C. Annu Rev Microbiol 1993;47:627–58.
[2] Woese CR, Kandler O, Wheelis ML. Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. Proc Natl Acad Sci U S A 1990;87:4576–9.
[3] Schönheit P, Schäfer T. Metabolism of hyperthermophiles. World J Microbiol Biotechnol 1995;11:26–57.
[4] Huber R, Huber H, Stetter KO. Towards the ecology of hyperthermophiles: biotopes, new isolation strategies and novel metabolic properties. FEMS Microbiol Rev 2000;24:615–23.
[5] Ishøy T, Kvist T, Westermann P, Ahring BK. An improved method for single cell isolation of prokaryotes from meso-, thermo- and hyperthermophilic environments using micromanipulation. Appl Microbiol Biotechnol 2006;69:510–4.
[6] Salerno M, Dante S, Patra N. Local increase in stiffness of agarose gel layer by patterning with polylysine measured via atomic force microscopy. J Mech Behav Biomed Mater 2010;3:425–35.
[7] Barros-Velázquez J, Jiménez A, Villa TG. Isolation and typing methods for the epidemiologic investigation of thermotolerant campylobacters. Int Microbiol 1999;2:217–26.
[8] Vartoukian SR, Palmer RM, Wade WG. Strategies for culture of 'unculturable' bacteria. FEMS Microbiol Lett 2010;309:1–7.
[9] Seale RB, Flint SH, McQuillan AJ, Bremer PJ. Recovery of spores from thermophilic dairy bacilli and effects of their surface characteristics on attachment to different surfaces. Appl Environ Microbiol 2008;74:731–7.
[10] Alain K, Querellou J. Cultivating the uncultured: limits, advances and future challenges. Extremophiles 2009;13:583–94.
[11] Seale RB, Bremer PJ, Flint SH, McQuillan AJ. Characterization of spore surfaces from a *Geobacillus* sp. isolate by pH dependence of surface charge and infrared spectra. J Appl Microbiol 2010;109:1339–48.
[12] Reneker DH, Yarin AL. Electrospinning jets and polymer nanofibers. Polymer 2008;49:2387–425.
[13] Lim SH, Mao HQ. Electrospun scaffolds for stem cell engineering. Adv Drug Deliv Rev 2009;61:1084–96.
[14] Satomura T, Kawakami R, Sakuraba H, Ohshima T. A novel flavin adenine dinucleotide (FAD) containing d-lactate dehydrogenase from the thermoacidophilic crenarchaeota *Sulfolobus tokodaii* strain 7: purification, characterization and expression in *Escherichia coli*. J Biosci Bioeng 2008;106:16–21.
[15] Fujita S, Ono D, Ohshima M, Iwata H. Supercritical CO_2-assisted embossing for studying cell behaviour on microtextured surfaces. Biomaterials 2008;29:4494–500.
[16] Bhardwaj N, Kundu SC. Electrospinning: a fascinating fiber fabrication technique. Biotechnol Adv 2010;28:325–47.
[17] Arima Y, Iwata H. Effect of wettability and surface functional groups on protein adsorption and cell adhesion using well-defined mixed self-assembled monolayers. Biomaterials 2007;28:3074–82.
[18] Tomasino C, Cuomo J. Plasma treatment — textiles. National Textile Center annual report; 1995. p. 241–7.