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Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) as an injectable implant system for prevention of post-surgical tissue adhesion

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ABSTRACT

An injectable implant system that immediately forms a film around the injection site of an animal was successfully developed by dissolving microbial polyester poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHx) in not harmful organic solvents including *N*-methyl pyrrolidone (NMP), dimethylacetamide (DMAC), 1,4-dioxane (DIOX), dimethyl sulfoxide (DMSO) and 1,4-butanolide (BL), respectively. The formation of the PHBHHx film was the result of contact between aqueous body fluids and the amphiphilic PHBHHx solvents, leading to the controllable precipitation (film formation) of PHBHHx around the contact site. The resultant PHBHHx film assumed the shapes of its surrounding cavities. The resulting porous PHBHHx film was not favorable for attachment of Human Embryo Lung Fibroblast (HELF) cells. As a consequence, the fibroblasts cultured on the PHBHHx film exhibited a spheroid-like morphology. It was found that hydrophilicity of the PHBHHx film prepared using the above technique was significantly reduced compared with the poly(lactic acid) (PLA) film prepared for the same purpose and a PHBHHx film prepared from chloroform casting. This reduced hydrophilicity explains the poor attachment of fibroblast cells to the injectable PHBHHx film, suggesting that the PHBHHx injectable implant system can be developed as a tissue adhesion prevention film for surgical operations.

1. Introduction

Biodegradable polymers have been used for many years for medical applications. For example, biodegradable sutures, surgical clips, staples, implants, and drug delivery systems. All instances involving their uses require the formation of stable polymeric structures outside the body, followed by insertion of the solid structures into the body. Their original shapes should be maintained over a certain period of time until the damaged tissues or cells recover their functionality [1,2].

Injectable in-situ forming implant systems have been developed based on biodegradable polymers [3]. Generally, an injectable in-situ forming system is prepared by dissolving a polymer in an organic solvent with non-toxicity and amphiphilicity to form a liquid polymer solution, this solution is then placed in a syringe and injected into the body when needed. Once in the body, the solvent dissipates in the aqueous body fluid, leaving the polymer behind to turn into a solid structure [4,5]. The implant will adhere to its surrounding tissues under mechanical force to assume the shape of its surrounding cavity [4,5].

Injectable in-situ forming implant systems have applications in a broad range of biomedical fields. Most commonly, the injection is performed like collagen to build up tissue or to fill the defects [4], sometimes for achieving drug sustainable delivery [3,6]. Dunn et al. developed a commercial system with a trade name of Atrigel®, in which both PLA and a drug are dissolved in an organic amphiphilic solvent, such as *N*-methyl-2-pyrrolidone (NMP) [6]. Although PLA is commonly used as a biodegradable polymer for an injectable in-situ forming implant system [7], it has disadvantages including rigidness, brittleness and too faster biodegradation that are not suitable for soft tissue applications [8,9].

Polyhydroxyalkanoates (PHA) are a class of polyesters produced by many bacteria and have attracted a lot of attention as bio-implant medical materials due to their degradability and non-harmfulness to surrounding tissues [10,11]. PHA and its composites have been used to develop various medical devices [12,13]. The TephaFLEX(R) Absorbable suture which derived from a type of PHA named poly-4-hydroxybutyrate (P4HB), was recently approved by FDA for surgical applications. Many studies conducted in our lab have revealed poly(3-hydroxybutyrate-co-3-hydroxyhexanoate)

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(PHBHHx), another member of PHA family, to have exhibited many advantages compared with PLA, poly-R-3-hydroxybutyrate (PHB), copolymer PHBV of R-3-hydroxybutyrate and R-3-hydroxyvalerate for medical applications [8–10,14,15].

In this study, we aimed to develop an in-situ implant system based on PHBHHx for possible application in tissue adhesion prevention for post-surgical applications.

2. Materials and methods

2.1. Materials

Copolyester PHBHHx ($M_w = 310{,}000$) consisting of 88 mol% R-3-hydroxybutyrate (HB) and 12 mol% R-3-hydroxyhexanoate (HHx) was donated by Microbiology lab of Tsinghua University. Polylactic acid (PLA) ($M_w = 120{,}000$) was purchased from Naturework (Minnesota, USA). *N*-Methyl pyrrolidone (NMP), dimethylacetamide (DMAC), 1,4-dioxane (DIOX) and dimethyl sulfoxide (DMSO) were obtained from Damao Chemical Reagent Co., Ltd (Tianjin, China). 1,4-Butanolide (BL) was from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Gibco-BRL (Gaithersberg, MD), fetal bovine serum (FBS) from Sijiqing Co., Ltd (Zhejiang, China). All reagents were of analytical grade.

2.2. Measurement of shear viscosity

PHBHHx and PLA were dissolved in organic solvents with concentrations of 12 wt%, 15 wt% and 17 wt% ($W_{polymer}/W_{polymer+solvent}$), respectively. The NDJ-1 rotary viscometer (NDJ-1, Shanghai Hengping Scientific Instrument Co., Ltd., China) was used to measure the viscosity of a polymer solution. The smallest rotor was selected with rotation rate maintained at 60 rpm. All operations were performed at room temperature.

2.3. Preparation of polymer films in vivo

To prepare PHBHHx or PLA polymer films, a 15 wt% polymer concentration in various organic solvents was considered appropriate. The solution was injected to the intra-abdominal position of SD rats by the aid of a syringe. A film was immediately formed when the solution diffused into the surrounding tissue and solvent diluted by the aqueous body fluids.

2.4. Preparation of polymer films in vitro

A PHBHHx or PLA solution with a concentration of 15 wt% was spread uniformly on a glass plate and then immersed immediately into water to allow polymer precipitation and film formation. The residual solvents were removed from the PHBHHx films by a series of washing steps. Finally, the films were dried in vacuum.

2.5. Scanning electron microscopy study of PHBHHx films

Morphologies of the porous PHBHHx films were examined under a scanning electron microscope (SEM) (JSM-6360LA, JEOL, Japan). Surfaces and cross sections of the films were coated with gold by means of a plasma multicoater. Subsequently, the samples were examined under the SEM.

2.6. Tensile strength testing

Tensile strength testing was performed on dumbbell-shaped samples (with a thickness 0.16–0.2 mm, base width 6 mm and base length 10 mm) cut from thin PHBHHx films at room temperature using a Universal Testing Machine CMT 4204 (Sans Co., Ltd., Shenzhen, China) with an extension rate of 5 mm min^{-1}. Measurements were performed at room temperature. Each value was the average of three parallel studies.

2.7. Surface contact angle measurement

The static water contact angle was measured at room temperature based on a sessile drop method using a telescopic goniometer (Model SL600; Shanghai Hengping Instrument and Meter Factory, Shanghai, China). Ten microliters of distilled water was gently dropped onto the surface of the films and the measurement time was less than 10 s. At least three readings on different parts of a film were averaged for data collection. The surface energy of tested film was determined by the contact angle of two types of reference liquids (H_2O and CH_2I_2) [16,17]. The surface free energy was calculated based on the Harmonic mean equations [18].

2.8. Cell culture

Human Embryo Lung Fibroblast (HELF) cells used in the experiments were donated by Guangzhou Institute of Biomedicine and Health of the Chinese Academy of Sciences (Guangzhou, China). Cell were cultured in DMEM with 10% FBS in an incubator at 37 °C under a humidified 5% CO_2–air atmosphere. To investigate the influence of the surface chemistry of a PHBHHx film on cell attachment, cells were seeded on the PHBHHx film.

2.9. Initial cell attachment

After the films were sterilized using 75% alcohol, they were washed three times with phosphate buffered saline (PBS). All films were transferred to 12-well plates, respectively. To avoid film floating on the wells, o-rings of glass tubings were used to keep the films attached to the bottoms of the wells. Approximately 1×10^4 HELF cells per well were incubated in serum-containing medium for 2, 6 and 10 h in 12-well plates. Subsequently, the supernatants were removed and the number of non-attached cells was counted with a hemocytometer. The percentage of adhering cells was calculated referring to initial cell concentrations. Each process was performed at least three times and the data were averaged.

2.10. Cell morphology

Fluorescein diacetate (FDA, Sigma–Aldrich, Taufkirchen, Germany) is a non-fluorescent dye that is internalized and converted to fluorescent product by cellular esterases in living cells only. To visualize the overall morphology of HELF cells, 5 ml FDA dissolved in acetone at a concentration of 5 mg/ml was added to each well. The samples were incubated for 2 min, washed with medium and mounted upside-down on glass slides with a drop of medium. The examination of cells was carried out immediately under a confocal laser scanning microscope (CLSM, LSM-510 META; Carl Zeiss, Jena, Germany).

2.11. Cell proliferation

Cell viability was assessed by the cell counting kit-8 (CCK-8 kit, Beyotime Biotechnology, Jiangsu, China). The method mentioned above was applied to treat the polymer films before seeding. HELF cells were seeded at an initial concentration of 1×10^4/well in a 12-well plate containing DMEM supplemented with 10% FBS. Cells seeded on NMP–PLA film were taken as a control group. After 24 h of cultivation, the culture medium was replaced with fresh medium. CCK-8 assay was performed to evaluate cell activity after 48 h of incubation [19]. Briefly, the culture medium was removed, approximately 900 μl serum-free DMEM medium and 100 μl CCK-8 solution were added to each sample, followed by incubation at 37 °C for 3 h. Supernatant was transferred to 96-well plate, the optical density (OD) at 450 nm and 630 nm was determined using a microplate reader (Multiskan MK3, Thermo Labsystems, Finland). 6 parallel experimental groups in each sample were used to assess the cell viability.

2.12. Statistical analysis

All data were presented as the mean value ± standard deviation (SD) of each group. Variation between groups was evaluated using Student's *t*-test, with a confidence level of 95% ($p < 0.05$) considered statistically significance and 99% ($p < 0.01$) considered very significant.

3. Results

3.1. Shear viscosity of polymer solutions

The shear viscosity of a polymer solution is very important for preparing an injectable liquid for the intended purpose in this study. At the same polymer concentration, the lower the viscosity, the better the injectable system. Following amphiphilic solvents that are used for *in vivo* products including *N*-methyl pyrrolidone (NMP), dimethylacetamide (DMAC), 1,4-dioxane (DIOX), dimethyl sulfoxide (DMSO) and 1,4-butanolide (BL) were selected for this study as they are the solvents for PHBHHx and PLA.

NMP–PHBHHx, DMAC–PHBHHx, DIOX–PHBHHx, BL–PHBHHx and DMSO–PHBHHx represent phase inversion of PHBHHx film prepared using *N*-methyl pyrrolidone, dimethylacetamide, 1,4-dioxane, 1,4-butanolide and dimethyl sulfoxide as a solvent, respectively. NMP–PLA indicates phase inversion of PLA film prepared in *N*-methyl pyrrolidone as a solvent. PHBHHx in DMAC and DIOX showed the lowest viscosity compared to other solvent

Table 1
Shear viscosity of various PHBHHx solutions compared with that of PLA dissolved in *N*-methyl pyrrolidone (NMP).

Samples	Viscosity (Pa s)		
	12 wt%[a]	15 wt%	17 wt%
NMP–PHBHHx	0.09 ± 0.01	0.18 ± 0.01	0.6 ± 0.1
DMAC–PHBHHx	0.03 ± 0.005	0.09 ± 0.01	0.2 ± 0.05
DIOX–PHBHHx	0.08 ± 0.001	0.15 ± 0.08	0.3 ± 0.05
BL–PHBHHx	0.3 ± 0.04	0.5 ± 0.01	0.76 ± 0.08
DMSO–PHBHHx	0.5 ± 0.06	0.82 ± 0.1	1.2 ± 0.1
NMP–PLA	0.9 ± 0.05	1.3 ± 0.2	3.1 ± 0.3

Rotary viscometer was used to measure the viscosity of the polymer solutions at room temperature. Viscosity was determined based on following equation: $\eta = k\alpha$ (η = viscosity, k = coefficient, α = angular deflection) (from instrument manual of NDJ-1 rotary viscometer).
NMP–PHBHHx, DMAC–PHBHHx, DIOX–PHBHHx, BL–PHBHHx, DMSO–PHBHHx represent phase inversion of PHBHHx film prepared using *N*-methyl pyrrolidone, dimethylacetamide, 1,4-dioxane, 1,4-butanolide and dimethyl sulfoxide as solvent, respectively. NMP–PLA indicates phase inversion of PLA film prepared in *N*-methyl pyrrolidone as a solvent.
[a] Polymer solution concentration ($W_{polymer}/W_{polymer+solvent}$).

systems with the same PHBHHx concentration (Table 1). They could be better systems for preparing injectable polymer gels for formation of films that could prevent tissue adhesion provided they showed suitable mechanical properties and not harmfulness to the surrounding tissues.

3.2. *In vivo PHBHHx film formation*

All gels containing PHBHHx in various organic solvents, respectively, formed a film when injected into intra-abdominal position of SD rats. The time of forming of a solid PHBHHx films appeared to be not different among all the solvent systems tested (Table 1). The resulting implant films were found to adhere to its surrounding tissue by mechanical force and could assume the shape of its surrounding cavity (Fig. 1).

3.3. *SEM study of PHBHHx film morphology*

PHBHHx films prepared from NMP, DMAC and DMSO showed larger porous structures both on surface and in the cross-section. Those from DIOX and BL had very small porous structures on the surfaces. In contrast, PHBHHx formed from chloroform casting had a smoother surface and smoother cross-section. Yet PLA from NMP revealed a wrinkle surface. The different surface morphologies could be attributed to the different solvent-exchange rate in phase inversion process involving organic solvents and aqueous liquid. Such a morphology could have an impact on the mechanical properties of the films formed, a suitable strength is required for preventing the adhesion of various tissues after the operation procedure. This was tested below.

3.4. *Wettability of PHBHHx films*

Tissue culture plate (TCP) has the most hydrophilic surface with the lowest water contact angle of 62° and the highest surface energy of 45 mJ/m^2 among all films tested (Table 2). Water contact angle of NMP–PHBHHx, DMAC–PHBHHx, DIOX–PHBHHx, BL–PHBHHx and DMSO–PHBHHx was 91.2°, 90.0°, 94.8°, 90.8° and 91.5°, respectively, which was significantly higher than films of NMP–PLA ($p < 0.01$). Chloroform cast PHBHHx films had a water contact angle of 86° which is in agreement with previous reported data [19]. Surface energies of all PHBHHx films were lower than that on TCP surface and NMP–PLA surface (Table 2), suggesting that surface microstructures including porosity may contribute to the reduced surface free energy (Table 2 and Fig. 2). These properties will affect the co-existence ability of the implant PHBHHx films [18].

3.5. *Mechanical properties of PHBHHx films*

Among all PHBHHx films prepared from various organic solvents, DIOX–PHBHHx film exhibited the highest tensile strength and tensile modulus of 8.0 and 489 MPa, respectively, with its elongation to break of 28%, second only to DMAC–PHBHHx (Table 3). In comparison, NMP–PLA was too brittle to be studied for its mechanical properties. NMP–PHBHHx film had the poorest mechanical properties in all tested PHBHHx films. Taking into consideration of porous surface and porous cross-sections, one can easily find that the poor mechanical strength of PHBHHx films was mainly due to these unique structures. For example, DIOX–PHBHHx had very fine surface and cross-section structures without many large porous formation, it thus appeared stronger than others with large porous structures (Fig. 2 and Table 3).

3.6. *Initial cell attachment to PHBHHx films*

Cell adhesion was determined at time intervals of 2, 6 and 10 h after seeding. In the first 2 h, BL–PHBHHx, DMSO–PHBHHx and the

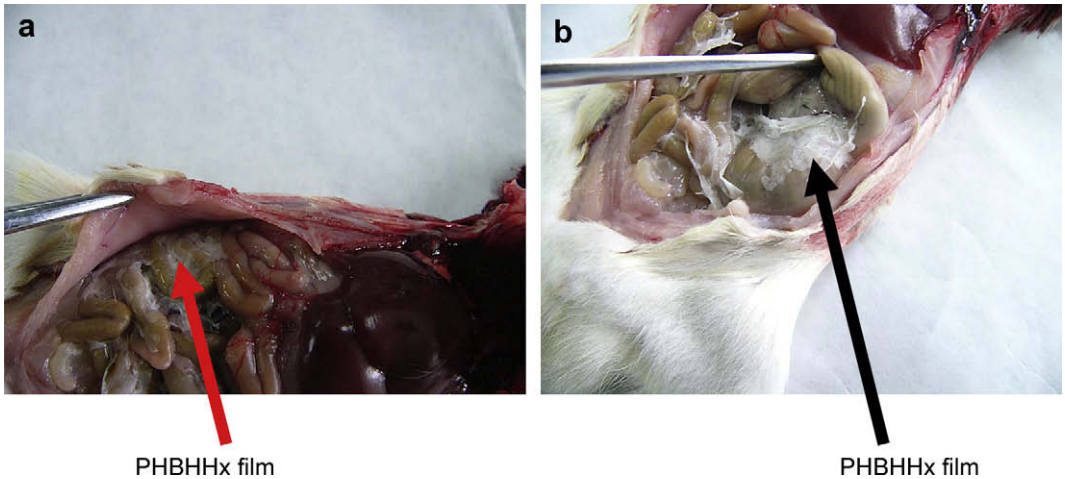

Fig. 1. *In vivo* formation of PHBHHx film when PHBHHx solution was injected into an animal (SD rat) PHBHHx solution was injected to the intra-abdominal position by the aid of a syringe. (a). Film of PHBHHx in DMAC formed below the peritoneal (b). Film of PHBHHx in DMAC form on the surface of the stomach.

Table 2
Water contact angles and surface free energy of various polymer films.

Samples	θ_{H_2O} (deg)	$\theta_{CH_2I_2}$ (deg)	Dispersive comp (γ_s^d)	Polar comp (γ_s^p)	Surface energy (γ_s) = (γ_s^d) + (γ_s^p)
NMP	91.2 ± 1.4*	34.6 ± 0.2	38.8 ± 1.0	4.1 ± 0.7	42.9 ± 0.3#
DMAC	90.0 ± 7.9*	39.2 ± 0.7	35.5 ± 1.7	5.1 ± 1.5	40.7 ± 0.5#
DIOX	94.8 ± 4.1*	39.0 ± 4.8	39.7 ± 6.8	2.8 ± 2.4	42.5 ± 4.4#
BL	90.8 ± 1.2*	43.3 ± 0.8	33.6 ± 1.0	5.1 ± 0.7	38.7 ± 0.4#
DMSO	91.5 ± 2.3*	53.6 ± 1.9	27.9 ± 0.7	6.0 ± 0.9	33.9 ± 1.0#
PLA	79.2 ± 1.2	38.0 ± 0.3	32.8 ± 2.5	10.2 ± 4.0	43.0 ± 1.6
Chloroform	85.6 ± 1.3	45.9 ± 0.3	31.4 ± 1.0	6.1 ± 0.4	36.6 ± 2.6
TCPs	62.3 ± 1.2	35.7 ± 0.9	26.2 ± 2.6	17.9 ± 0.6	45.0 ± 2.5

NMP: *N*-methyl pyrrolidone; DMAC: dimethylacetamide; DIOX: 1,4-dioxane; DMSO: dimethyl sulfoxide; BL: 1,4-butanolide.
Data presented as mean ± standard deviation ($n = 3$ for each example). θ_{H_2O}: contact angles of H_2O. $\theta_{CH_2I_2}$: contact angles of CH_2I_2. Comp: component.
NMP have same meaning as NMP-PHBHHx described in Table 1. This rule is also applied to DMAC, DIOX, BL, DMSO, PLA.
Chloroform indicates chloroform cast PHBHHx film.
TCPs: tissue culture plates.
*$p < 0.01$ compared to PLA control.
#$p < 0.05$ compared to PLA control and choloroform cast film group.

control NMP–PLA were found to be adhered with more cells than NMP–PHBHHx, DMAC–PHBHHx and DIOX–PHBHHx. After 6 h of incubation, only NMP–PLA was approximately 20% better than other PHBHHx films that had narrowed their difference in cell adhesions (Fig. 3). At 10 h, no difference was found among all PHBHHx films for cell adhesion. We concluded that all PHBHHx films showed similarly poor cell adhesion properties.

3.7. Study of morphology of cells grown on various PHBHHx films via confocal laser scanning microscopy (CLSM)

When HELF cells were cultured on various PHBHHx films for 48 h, many of their morphology appeared to be small and spherical in size for those on NMP–PHBHHx, DMAC–PHBHHx, DIOX–PHBHHx and BL–PHBHHx, respectively. In contrast, cells grown on NMP–PLA, chloroform cast PHBHHx film and TCP showed extended shapes with longer and larger sizes (Fig. 4). At the same time, it could be noticed that the number of cells was not significantly different on various PHBHHx films. This result was consistent with that of the CCK-8 assay (Fig. 5).

3.8. Viability of cells grown on various PHBHHx films

CCK-8 assay was performed to evaluate cell viability. After 48 h of incubation, the viability of HELF cells grown on NMP–PHBHHx, DMAC–PHBHHx, DIOX–PHBHHx, BL–PHBHHx and DMSO–PHBHHX films was 63%, 61%, 67%, 61% and 55% of those on TCP, respectively (Fig. 5). No significant difference was observed among the above PHBHHx films on cell viability. Interestingly, viability of cells grown on chloroform cast film was 73% of those on TCP. The highest viability of 80% of TCP cell viability was found for cells grown on NMP–PLA film (Fig. 5). Significant difference on cell viability was found between PHBHHx films and PLA one. Therefore, the selection of suitable PHBHHx injectable implant system for prevention of tissue adhesion must take into consideration of cell viability, film mechanical properties, surface properties and injectability together.

4. Discussion

Most of injectable adhesion prevention implants use PLA as a polymer in various solvents [7]. So far, no study has been reported using PHBHHx based injectable gels for implanting tissue adhesion prevention films serving post-operation procedure.

As an injectable implant system, it is necessary that the system consisting of a polymer and a solvent is non-toxic, floating with lower viscosity, *in vivo* biodegradable with sufficient mechanical properties when solidified into a film *in vivo*. The solvent for the biodegradable polymer should also be non-toxic, water miscible and not harmful to tissues as approved by US FDA for *in vivo* applications [4]. The solvent used in this study must dissolve PHBHHx easily, it should allow a quick formation of PHBHHx film when injecting into the body. Many implant products containing organic solvents have been approved by FDA, such as NMP and DMSO that have been used in commercial injectable products trade named as Atrigel® [6] and PERIOceutick™ gel [20] for human applications. In this study, NMP, DMAC, DIOX, BL and DMSO have been proven to have low toxicities as evidenced by intraperitoneal LD50 value in rats of over 2472 mg kg^{-1} for NMP, 1400 mg kg^{-1} for DMAC, 790 mg kg^{-1} for DIOX, 1000 mg kg^{-1} for BL and 8200 mg kg^{-1} for DMSO (see: Material Safety Data Sheet from physical and theoretical chemistry laboratory of Oxford University). These organic solvents can dissolve PHBHHx, thus, they were considered to be used for injectable PHBHHx system in this study.

The viscosity of a polymer solution is one of the important factors for an injectable implant system as it is related to storage stability and injectability [21]. Properties of a polymer and the polymer concentration affect the viscosity of a polymer solution. In addition, viscosity of a polymer solution increases with increasing polymer concentration (Table 1). Based on the relationship between viscosity and concentration, the concentration of the resulting polymer solution must be low enough to allow syringe injection. However, a low polymer concentration results in poor mechanical property when a thin polymer film is formed *in vivo*, this is not desirable for surgical operation. Injectability test showed PHBHHx gel has an excellent film-forming ability when PHBHHx concentration is equal to or higher than 15% in its solvent. 15% PHBHHx gel was injectable, it quickly formed a film with proper strength around the injection site. Combining the suitable injectability and appropriate mechanical property of the resulting film, 15% PHBHHx was taken as an appropriate concentration for preparing the PHBHHx gel to all studies (Figs. 1–5 and Tables 2 and 3). Depending on solvents used, viscosity varied, ranging from 0.09 to 1.3 Pa/s, all of these viscosity did not prevent the 15% PHBHHx to become injectable (Table 1).

To address cell responses to PHBHHx implant, PHBHHx films were prepared from different solvents with the addition of aqueous solvent, which is similar to film formation *in vivo* after injection of a PHBHHx gel. It was found that different tested films exhibited different cell adhesions and cell viability (Figs. 3–5). HELF cells grown on various PHBHHx film showed a similar viability although they were not as active as their growth on PLA film and on TCP (Fig. 5). This result is different from previous studies indicating PHBHHx had better tissue compatibility than that of PLA [19,22], this could be due to the differences of surface structures of the PHBHHx prepared in this study which had low hydrophilicity compared with that of chloroform cast PHBHHx film. HELF cell viability on all PHBHHx films prepared from the five solvents (not including the chloroform cast PHBHHx film) was also shown to be similar by the initial cell attachment and morphology observation (Figs. 3 and 4). On all PHBHHx films, the number of initial cell attachment was poor, and the cells were spherical, indicating the cells were not ready for proliferation (Fig. 4). However, cells incubated on PLA, chloroform cast PHBHHx films and TCP had changed their morphology to longer and bigger structures together with an obvious increase in number, suggesting they were in the growth status (Fig. 4F–H). This was consistent with the results of CCK-8 assay (Fig. 5), implying that PHBHHx films formed from the

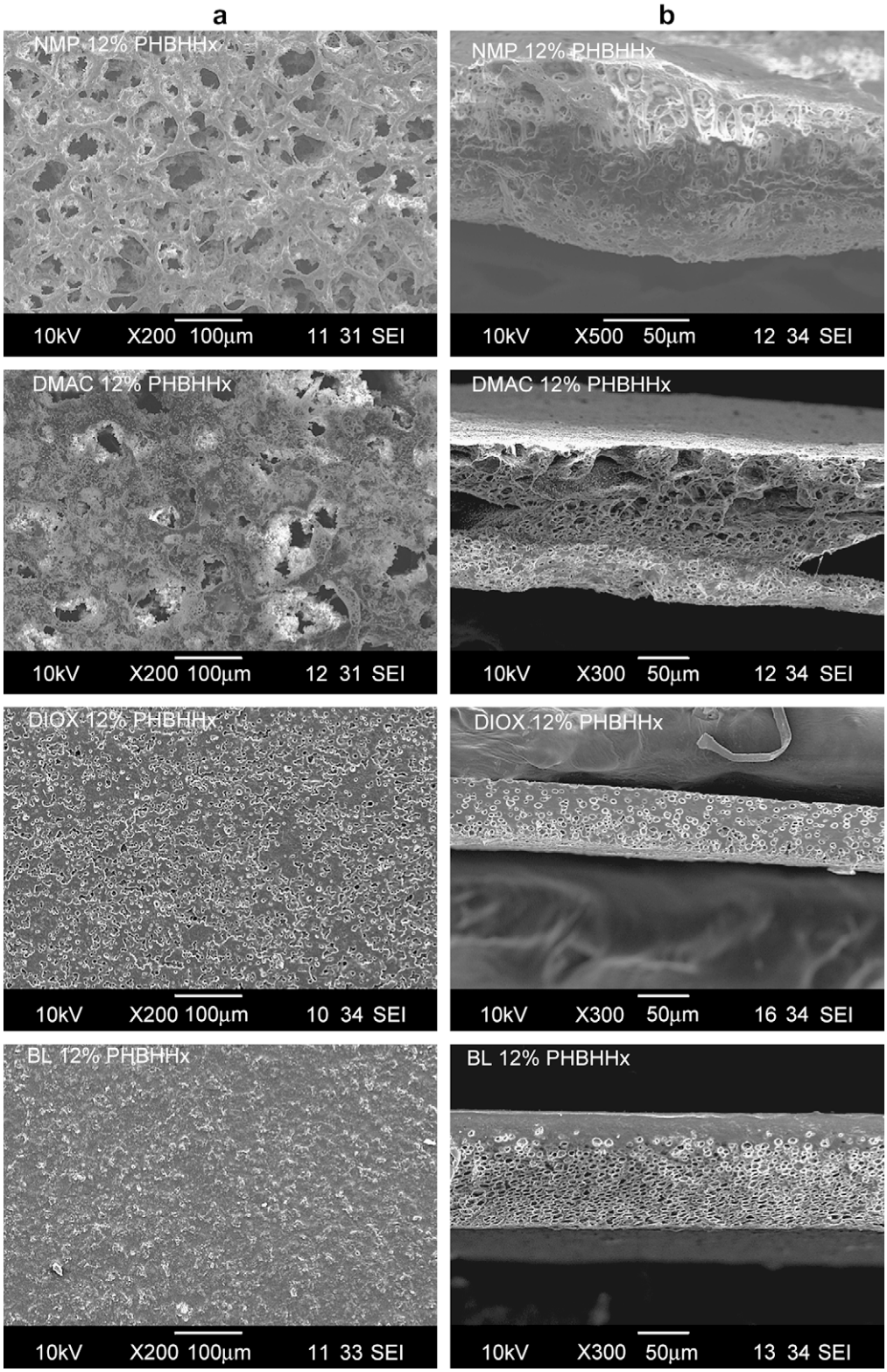

Fig. 2. Scanning electron microscope study of PHBHHx films. PHBHHx films were obtained via either aqueous fluid precipitation or solution casting. The films formed were dried under *vacuum before SEM examinations*. (a). Surface of the various PHBHHx films (b). Cross sections of various PHBHHx films.

amphiphilic solvents were neither favorable for cell attachment nor for cell growth (Figs. 4 and 5).

Surface and cross-section morphologies of a polymer film are influenced by polymer concentrations, molecular weights, crystallization degrees, solvents, miscibility between solvent and non-solvent [4,23]. PHBHHx films prepared using different solvents via phase inversion method exhibited different porous structures (Fig. 2), this was reported to be the results of film formation process [24,25]. On the other hand, surface microstructure affects surface hydrophilicity [26,27]. As a result, PHBHHx films prepared from all amphiphilic

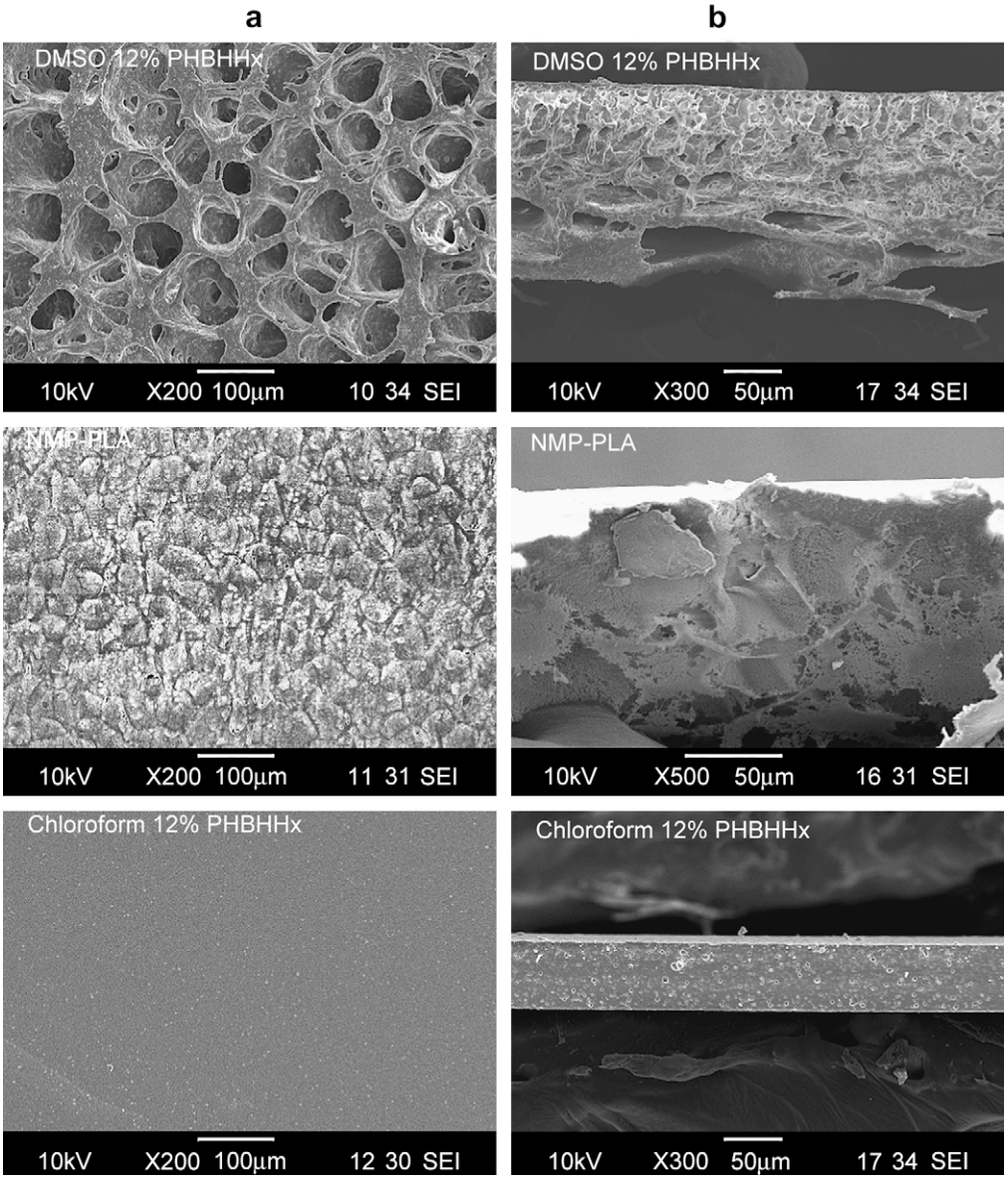

Fig. 2. (*continued*).

solvents possessed a larger water contact angle compared with that of the chloroform cast PHBHHx film, and thus they were more hydrophobic than chloroform cast PHBHHx film, PLA and TCP (Table 2). The increasing hydrophobicity of the porous PHBHHx films prepared from amphiphilic solvents explains partially the poor cell attachment and its resulting tissue adhesion prevention effect.

It has been reported that the surface chemistry of a material can mediate the cellular response to the material and, in turn, affect cellular function on the surface including cell adhesion, proliferation and migration [28,29]. Surface wettability was the most important factor [9]. Studies on relationship between cell adhesion and water contact angle for a variety of polymer surfaces revealed that materials whose contact angles higher than 90° did not favorably allow cell adhesion [30]. Studies on the influence of surface energy on cell attachment and spreading further showed that cells exhibited good attachment and spreading on a materials surface with a high surface energy [31]. The contact angles of all PHBHHx films from amphiphilic solvents are higher than 90°, the order of their contact angles is NMP–PLA film < chloroform casting PHBHHx film < all PHBHHx films from amphiphilic solvents (Table 2). HELF cells on films with high contact angles and low surface free energy (compared with that of PLA and TCP) exhibited poor proliferation and spherical morphology, suggesting the suitability for the PHBHHx films to be used as anti-adhesion films (Figs. 3–5).

Table 3
Mechanical property of tested films.

Samples	Tensile strength (MPa)	Tensile modulus (MPa)	Elongation to break (%)
NMP–PHBHHx	0.08 ± 0.02	12.95 ± 1.2	0.75 ± 0.13
DMAC–PHBHHx	0.54 ± 0.1	24.78 ± 2.0	72.33 ± 6.4
DIOX–PHBHHx	8.00 ± 1.6	488.95 ± 20.5	27.77 ± 5.4
BL–PHBHHx	1.08 ± 0.4	27.98 ± 4.6	21.15 ± 9.3
DMSO–PHBHHx	0.17 ± 0.06	13.63 ± 1.02	31.42 ± 3.6
Chloroform–PHBHHx	6.15 ± 1.3	311.73 ± 10.3	10.54 ± 2.1

NMP: *N*-methyl pyrrolidone; DMAC: Dimethylacetamide; DIOX: 1,4-dioxane; DMSO: Dimethyl sulfoxide; BL: 1,4-Butanolide.
NMP–PHBHHx et al. represents a phase inversion of PHBHHx film prepared using *N*-methyl pyrrolidone as described in Tables 1 and 2.
Data for NMP–PLA film is not showed here since the PLA film was too brittle to be studied for mechanical properties.

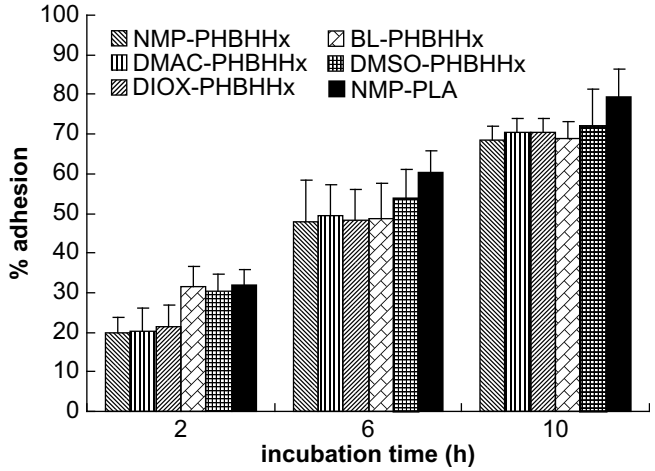

Fig. 3. Percentage of HELF cells adhering to various PHBHHx films compared with that to the NMP–PLA film over the incubation periods of 2, 6 and 10 h. Approximately 1×10^4 HELF cells per well were incubated in serum containing medium for 2, 6 and 10 h in 12-well plates containing various PHBHHx films, respectively. To count the cells, supernatants were removed and the number of non-attached cells was counted using a hemocytometer. A percentage of adhering cells was calculated referring to an initial cell concentration. Each data was the average of at least three parallel samples.

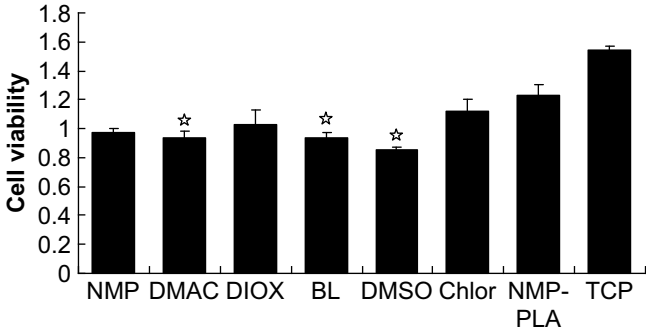

Fig. 5. Viability study of HELF cells incubated on various polymer films prepared by phase inverse method via CCK-8 assay. All tested films were transferred to 12-well plates. Cells with an initial concentration of 1×10^4/well were seeded in various wells on various plates. Cells incubated in NMP–PLA were taken as a control. CCK-8 assay was performed to quantitatively evaluate viability of cells incubated for 48 h in DMEM supplemented with 10% FBS. All CCK-8 assays were performed using six parallel samples. $p < 0.05$ vs. NMP–PLA group.

Although surface energy of chloroform cast PHBHHx film was observed among the lowest of all films tested, cells grew better on it than that of other PHBHHx films from amphiphilic solvents (Table 2 and Fig. 5). This could be due to the stronger hydrophilicity of the chloroform cast PHBHHx film which plays a dominant role for cell attachment. Results obtained via the contact angle study were in good agreement with the data of above cell experiment (Table 2 and Fig. 5). It was therefore proposed that PHBHHx films from amphiphilic solvents are not able to induce cell attachment and morphological change of cells incubated on them.

Adhesion formation is a natural consequence of surgery when the damaged tissue begins to heal. However, adhesion can cause serious complications for a patient including small bowel obstruction, female infertility, chronic debilitating pain and difficulty for future operations, etc. [32,33]. It is very important to prevent post operational tissue adhesions since tissue adhesions tend to recur once they happen even after they are surgically removed [33]. When peritoneum is injured by trauma, leakage of plasma proteins from damaged surfaces forms a bridge between the damaged surfaces and surrounding tissues. In the initial phases of adhesion formation, the process can be reversed by enzymatic degradation via locally released fibrinolysins [34,35]. Trauma decreases activity of fibrin splitting enzyme, therefore, it decreases peritoneal fibrin-clearing capacity. Within five days the fibrin mesh is invaded by proliferating fibroblasts, which replace the fibrin with more durable components of the extra-cellular matrix, such as collagen [34].

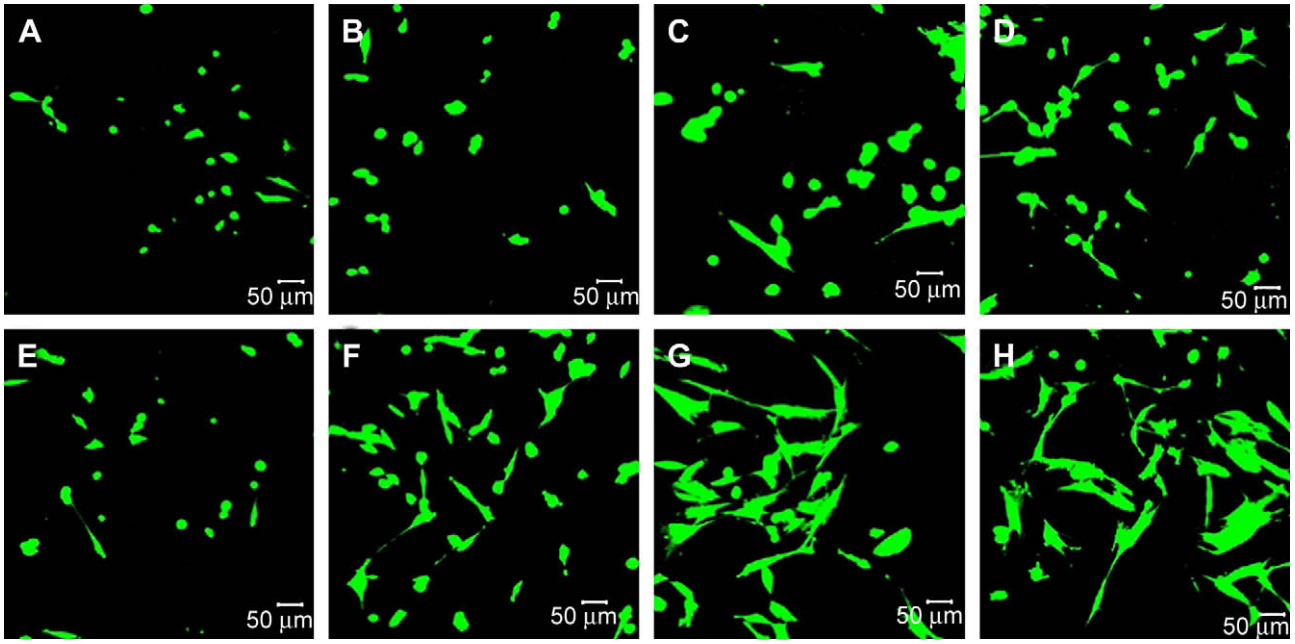

Fig. 4. Growth and morphology of HELF cells stained with FDA after 48 h of incubation on different polymer films under a laser confocal microscopy. Approximately 1×10^4 HELF cells per well were incubated in serum containing medium for 2, 6 and 10 h in 12-well plates containing various PHBHHx films, respectively, as described in Fig. 3. (A) NMP–PHBHHx, (B) DMAC–PHBHHx, (C) DIOX–PHBHHx, (D) BL–PHBHHx, (E) DMSO–PHBHHx, (F) NMP–PLA, (G) Chloroform casting PHBHHx film, H. TCP (Tissue culture plate). Bar = 50 μm.

The use of barrier films during surgery such as "interceed", "seprafilm" to protect raw tissue surface when they heal have been shown to be one of the most effective methods of reducing adhesion [36,37]. Adhesion prevention will be evident when a film inhibits fibroblast proliferation [38,39]. Yamaoka et al. had demonstrated that materials which are not conducive to adhesion of cells show promises as adhesion-prevention product [38]. Research also revealed that adhesion prevention mechanism of chitosan is to reduce fibroblast adhesion [39].

Wang et al. revealed that PHBHHx films were biodegraded easily [40]. In this study, porous structures of the PHBHHx films increased the surface area, which should result in even faster degradation. It is generally believed that the first seven days after an operation is key for adhesion prevention [33]. Since PHBHHx biodegradation is slower than that of PLA [41], it is believed that PHBHHx films could at least maintain the integrity for the first seven days for adhesion prevention.

To sum up, the use of PHBHHx based implant system as part of surgical technique offers promises for adhesion prevention. This implant system can be injected via a syringe into the body. Once injected, PHBHHx is solidified to form a stable film which is applied to the surfaces of targeted tissues during surgery, it should be stable for at least seven days post operation as PHBHHx is more stable than PLA. Subsequently, the PHBHHx material can be biodegraded *in vivo* and is therefore naturally cleared from the body [32–34].

5. Conclusion

In this study, an injectable in-situ film forming polymer gel was developed by dissolving 15 wt% PHBHHx in non-toxic and amphiphilic solvents. Films with porous structures were formed when the gel was injected and came into contact with aqueous body fluid. Films prepared from 1,4-dioxane (DIOX) showed the strongest mechanical properties among all PHBHHx films studied. All the PHBHHx films from amphiphilic solvents were found not favorable for HELF cell attachment and proliferation compared with the traditionally used PLA films for the same purpose. It was concluded that PHBHHx based injectable system can be further developed into a new adhesion prevention product.

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Appendix

Figures with essential colour discrimination. Certain figures in this article, in particular parts of Figures 1 and 4, may be difficult to interpret in black and white. The full colour version can be found in the on-line version at doi:10.1016/j.biomaterials.2009.02.015.

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