Fabrication of Sustained-release CA-PU Coaxial Electrospun Fiber Membranes for Plant Grafting Application

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ARTICLE INFO

Article history:
Received 7 February 2017
Received in revised form 5 April 2017
Accepted 10 April 2017
Available online 12 April 2017

Keywords:
Coaxial Electrospun
cellulose acetate
polyurethane
Callus proliferation
Plant grafting

ABSTRACT

Plant grafting is a well-known activity in orchards, greenhouses and vineyards gardens. However, low survival rate still limits the promotion of grafting and breeding of improved varieties. We report on polymeric fibers, obtained through coaxial electrospun, as carriers for the sustained release of drugs to heal wounds in plants. The CA-PU co-electrospun fibers show a rather uniform diameter, a smooth and hydrophilic surface. As long as 10 days of sustained drugs release meets with the physiological requirement of plant grafting. The callus toxicity test shows that the CA-PU fibers are not toxic for plant cells. We show that loading the core of CA-PU fibers with 6-Benzylaminopurine (6-BA), a first-generation synthetic cytokinin that elicits plant growth and development, results in fibers that can efficiently promote the healing of plant wounds, thereby significantly improving the grafting survival rate.

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Introduction

Plant grafting has been widely used in orchards, greenhouses, vineyards and gardens. One possibility is grafting the shoot of one plant (the scion) to the root of another plant (the rootstock)\cite{Kuzbota, Mcclure, Koka, Bausher, Notaguchi, 2016a}. Grafted plants often offer some advantages compared with their parents. For example, by plant grafting one can obtain plants which show improved stress resistance\cite{King, Davis, Liu, 2008} or which can grow in new environments. Heavy-cropping shoots can be grafted onto robust rootstock. In addition, grafting has been done successfully not only within the same species, genus and family, but even among different families\cite{Notaguchi, 2016a, 2016b}. Although plant grafting has been practiced for over 2,500 years, the low survival rate, which is typical as low as ~30\% \cite{Greenwood, 2010}, remains a limitation. Typically, exogenous auxins are added manually by treating scion impregnated with a solution containing auxin which causes insufficient auxin and mismatches the needs of existing masteries\cite{Edelstein, 2004}. Therefore, there is great interest in designing plant grafting materials which have the potential which slowly release exogenous auxins into the plants.

In recent years electrospun fibers have been intensively investigated for various purposes\cite{Ma, Zhu, 2016}. On the one hand electrospun fibrous scaffolds have a high surface-to-volume ratio\cite{Nie, Wang, He, 2016} and high porosity features being favorable for e.g. cell adhesion\cite{Cui, Zhou, Chang, 2016; Jiang, Carbone, Lo, Laurencin, 2015}, cell migration\cite{Huang, 2011}, cell proliferation \cite{Huang, 2012b} while the high porosity of electrospin mats aids in nutrient transport. On the other hand electrospin fibers show potential application in drug delivery\cite{Huang, 2012a; Yuan, 2015} for wound treatments\cite{Yang, Mun, Kim, 2015}. Such fiber based drug delivery vehicles may tremendously reduce the systemic absorption of drugs, as well as provide localized therapeutic effect at low dose of drugs.

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In this work, we aimed to design electrospun fibers, based on polymer cellulose acetate (CA) and polyurethane (PU), as carriers for the sustained release of plant growth promoting compounds to improve plant tissue wound healing and thus improving the survival rate of grafting. Although electrospun CA fibers are biocompatible (Jia et al., 2013; Rodríguez, Gatenholm & Renneckar, 2012), their poor mechanical performance could limit their application as plant wound dressings. To overcome this, the current work focuses on the design of core-shell fibers obtained through coaxial electrospun (Hua et al., 2016). To enhance the mechanical properties of the fibers, a core is fabricated by polyurethane (PU) which possesses excellent mechanical properties (like high elongation at break and high mechanical strength (Jiang, Duan, Zussman, Greiner & Agarwal, 2014; Jiang, Greiner & Agarwal, 2013)) due to the thermodynamic incompatibility between soft and hard segments in the PU main chains (Hong, Guan, Fujimoto, Hashizume, Pelinescu & Wagner, 2010; Skarja & Woodhouse, 1998). The shell is made from CA which is widely used in pharmacy (Rubenstein et al., 2010). Core-shell fibers have been investigated to achieve sustained drug release (Qi et al., 2010), e.g. for slow release of microbicides (Ball, 2014). Inspired by the excellent features of CA-PU core-shell fibers, we hypothesize that CA-PU core-shell fibers is potential to be used as membrane dressing in plant grafting. Here, we first report on the fabrication and characterization of electrospun CA-PU fibers (membranes). Next we validate the release of 6-BA from the fibers and evaluate their toxicity. Finally, we report on how such dressings perform in respectively plant wound healing and plant grafting.

1. Materials and methods

1.1. Materials

Rhodamine B, Polyurethane (PU, Mw = 100,000 g/mol), Tetrahydrofuran (THF), N,N-Dimethylformamide (DMF) and Acetone were purchased from Sino pharm (Shanghai, China). Cellulose acetate (CA, Mw = 60,000 g/mol) was purchased from Sigma Aldrich (Steinheim, Germany), 6-Benzylaminopurine (6-BA) was purchased from Mumu Science and Technology (Hangzhou, China).

1.2. Electrospun CA, PU and CA-PU fibers

PU (core)-CA (shell) fibers were fabricated by coaxial electrospun a CA solution (12%, wt./v; N, N-Dimethylformamide (DMF)/acetone (1:2, v/v) as solvent) and a PU solution (15%, wt./v; THF/DMF (1:1, v/v) as solvent). Rhodamine B was added to the PU solution prior to electrospun. For coaxial electrospun the CA and PU solutions were filled in two individual syringes, a coaxial electrospun needle (0.4 mm (inner), 0.9 mm (outer)), while the flow rate equals respectively 0.8 ml/h for the CA solution and 0.6 ml/h for the PU solution. The needle was connected to a high-voltage power supply, the positive DC voltage was fixed at 16 kV. An aluminum foil covered metal plate was horizontally placed to collect the fibers with a needle tip-to-plate distance of 15 cm. The fibers were collected on a metal drum plate (d = 30 cm) which operates at a speed of 1000 r/min. The ambient temperature (25 °C) and humidity (65%) were controlled. As a control, ‘pure’ CA and PU electrospin fiber mats were prepared under the same conditions (except that the needle is a single channel). All electrospun fiber membranes were vacuum dried during 24 h at 40 °C.

1.3. Characterizations of electrospun fibers

To visualize the electrospun fiber membranes, they were first coated with a thin layer of gold using a sputter coater before they are examined by a scanning electron microscope (SEM). The images were captured using a field emission SEM instrument (JSM-7600F, Japan) operated at an acceleration voltage of 5 kV. TEM images were captured with a JEM-2100 (Japan) transmission electron microscope. The acceleration voltage was 100 kV. Thermogravimetric Analysis (TGA) of the electrospun fiber membranes was performed in air using a temperature sweep from 30 °C to 800 °C at 10 °C/min (V4.5A TGA instrument). The tensile tests were carried out by a universal tensile test machine equipped with a load cell which has a maximum load of 50 N with a resolution of 0.001 N. The samples (50 mm * 5 mm) were stretched at a speed of 5 mm/min while the gauge length is set to 25 mm. The average thickness of the samples was measured making use of a screw micrometer. Fourier transform infrared (FT-IR) spectra were recorded with a VERTEX 80 v FTIR spectrometers. X-ray diffraction (XRD) patterns were obtained on a D/Max-B diffractometer (Rigaku, Tokyo, Japan) with CuKα radiation within the 20 range of 5°–90°.

1.4. Rhodamine B release

Rhodamine B loaded fibers were electrospun and vacuum dried at room temperature; Therefore 2.4 mg Rhodamine B was dissolved in 10 ml CA solution (solvent composition see above) used for electrospin (0.2%, R–Bwt./CAwt.). 2 mg of Rhodamine B loaded fiber mats were dispersed 10 ml of deionized water (which served as ‘Plant body fluids’). The release of Rhodamine B from the fibers was studied by UV–vis (at 551 nm), Rhodamine B standard curves (in deionized water; concentration range between 0.001 and 0.01 mg/ml) were used to determine the concentration of the released Rhodamine B.

1.5. Contact angle measurements

The contact angle measurements were carried out using a contact angle measurement setup (JC2000D2 Contact angle measuring instrument, Shanghai) in static/dynamic sessile drop mode at room temperature.

1.6. Toxicity evaluation of callus

CA-PU membranes (2 cm * 2 cm), respectively without 6-BA or loaded with 6-BA (see under Section 2.7) were prepared. And first ultraviolet light irradiation for 8 h, followed by 75% ethanol solution for 15 minutes, and finally rinsed through sterile water, retained. The toxicity of the membranes was evaluated by the somatic embryogenesis system of immature embryo derived from Liriodendron tulipifera. Using MS (Tissue culture a liquid) medium(Zhi, Bian-Li, Wu & Shi, 2007; Jinhu, 2003), the callus of Liriodendron tulipifera was placed on the membranes and compared with the non-basal material and incubate at 24 degrees Celsius, three sets of parallel experiments were performed and the callus are observed after 10 days.

1.7. Auxin membrane preparation and its application

0.24 mg and 2.4 mg 6-Benzylaminopurine (6-BA) was dissolved in 10 ml PU solution (which corresponds to 0.02% and 0.2% 6-BAwt./PUwt.). 70 cm * 2 cm membranes were prepared. The Chinese ornamental plant Liriodendron chinense was used as test plant. Firstly, an incision (4 mm*8 mm) was made in the branches and subsequently wrapped with respectively traditional membranes (Polyethylene), ‘pure’ CA-PU co-axial electrospin membranes and CA-PU co-axial electrospin membranes (containing 0.02%wt and 0.2%wt 6-BA in the core of PU, respectively). Each kinds of membranes tested for twenty and repeated for three times indenpendently. And observed after ten days healing. On the other hand, we did a plant grafting to characterize of its overall performance. Pyrus
betulifolia Bunge as rootstock and Pyrus calleryana Decne as scion was used in this experiment.

2. Results and discussion

2.1. Morphology of CA-PU coaxial electrospun fibers

To provide the ‘bio-friendly’ CA electrospun fiber with excellent mechanical property (Jia et al., 2013; 2012), we fabricated CA-PU core-shell electrospun fibers as shown Fig. 1. Fig. 1A and B shows the morphology of respectively CA (0.02%wt 6-BA) fibers and PU fibers (zoomed-in pictures in Fig. 1D and E) with an average diameter of 960 ± 470 nm and 950 ± 230 respectively (Fig. 1G and H). Both types of fibers show a smooth surface while PU-fibers seem highly curled can play a better seal. The morphology of coaxial electrospun CA-PU (containing 0.02%wt 6-BA in the core of PU) fibers can be seen in Fig. 1C (SEM, zoomed-in picture in Fig. 1F) with a narrow diameter distribution and an average diameter of 380 ± 170 nm (Fig. 1I). The images of ‘pure’ CA and ‘pure’ CA-PU fibers are provided in the supporting information of Fig. S1 with an average diameter of
920 ± 380 nm and 310 ± 150 respectively (Fig. S1 E and S1F). With the addition of 6-BA, the incorporation of 6-BA in the polymeric solution leads to increase the fiber diameter probably due to the increase in viscosity. The TEM image is shown in Fig. 1J, the core and the shell of the fibers can be distinguished clearly. Importantly, the PU-core is completely wrapped by the CA-shell. The TGA data in Fig. 1K show that CA-PU fibers remain stable upon heating as long as the temperature remains below 300 °C. It suggests that such electrospun membranes material can be sterilized at high temperature which is attractive for many applications.

2.2. The physical status of the components in the nanofibers

As shown in Fig. 2, the absorption peaks of 6-BA around 1621 cm⁻¹ could be assigned to the ending vibration of –NH₂. The absorption peaks of CA electrospun fibers around 1740 and 1040 cm⁻¹ could be assigned to C=O and the wagging of C–O, respectively. In comparison with CA, the spectrum of PU electrospun fiber displays peaks at 1729 and 1529 cm⁻¹, which correspond to the absorption of C=O and the wagging of N–H, respectively. The absorption peaks of CA-PU co-electrospun fibers at 1730, 1529 and 1040 cm⁻¹ are assigned to the stretching vibration of C=O, N–H and C–O, respectively, indicating successful electrospun of CA-PU co-electrospun fibers. However, all the peaks for 6-BA disappeared in the FTIR spectra of the composite nanofibers CA-PU (0.02%wt 6-BA) fibers compare with the pure CA-PU fiber. Only one large peak at 1730 cm⁻¹ for composite nanofibers is noted, which may contributes to the small amount of the auxin added. 6-BA. CA molecules possess free hydroxyl groups (–OH) and these could act as potential proton donors for hydrogen bonding. CA and PU molecules have carbonyl groups (–C=O) and so could act as proton acceptors. Therefore, it can be speculated that hydrogen bonding interactions can occur within the composite fibers through interactions between these groups. However, there is no free hydroxyl groups (–OH) and –C=O in the 6-BA molecules, so the intermolecular forces dominate.

A slight white powder to the naked eye (Fig. 3b insert image), comprised crystals in the form of prisms or needles, and revealed a rough surface under Optical microscope (Fig. 3b). The presence of many distinct peaks (Fig. 3a) in the XRD pattern of pure 6-BA similarly verified that 6-BA is present as a crystalline material. The diffraction patterns of raw CA powders exhibit a diffuse background pattern with one diffraction halo, indicating that the polymer is amorphous. The spectrum of amorphous PU was characterized by the complete absence of any diffraction peak. From Fig. 3, it is clear that the characteristic diffraction peaks of crystalline 6-BA are completely absent for the CA-PU co-electrospun fibers (containing 0.02%wt 6-BA in the core of PU). These results suggest that all the 6-BA in the CA-PU co-electrospun fibers are amorphous and are evenly distributed.

2.3. Wettability of CA-PU co-electrospun fiber membranes

For plant wound healing, membranes should show a sufficient for water (wettability). CA is an insoluble and hydrophilic polymer and 6-BA is a poorly water-soluble active pharmaceutical ingredient. As shown in Fig. S2, the contact angle of ‘pure’ CA and ‘pure’ PU membranes are 45.1° and 91.3°, respectively. The contact angle of ‘pure’ CA-PU co-electrospun membranes is 45.7° which is very close to the value of the ‘pure’ CA membranes, confirming the core-shell architecture of the CA-PU fibers. With the addition of 6-BA, the contact angle of the CA and CA-PU (containing 0.02%wt 6-BA) electrospun fiber membranes change from 45.1° and 45.7° to 45.4° and 46.1° (Fig. 4). There is a little increase, but still has a good hydrophilic.

2.4. Mechanical properties of CA-PU coaxial electrospun fibers

Although CA fibers are considered to be bio-friendly, they suffer from poor mechanical properties. On the contrary, PU electrospun fibers have been reported to show excellent mechanical properties i.e. a high (critical) tensile strength and a high elongation at break of 589%, suggesting PU electrospun fibers to be good reinforcements and toughening materials. As shown in Fig. 5A and B, CA and ‘pure’ PU electrospun fiber membranes show a critical
tensile strength of 8 ± 0.5 and 28 ± 1.0 MPa, respectively. The critical tensile strength of CA-PU co-electrospun membranes reaches 12.5 ± 0.5 MPa which is a clear improvement when compared to that of CA fibers. CA electrospun membranes show an elongation at break of about 1.6%. Incorporating PU leads to a significant increase of the elongation at break up to 10%. (Fig. 5A and B). Compared with the ‘pure’ fiber membrane without the addition of 6-BA shows in Fig. S3, the mechanical properties of the electrospun fiber membrane were not significantly altered, which may be due to the small amount of the auxin added.

2.5. Rhodamine B release of CA-PU coaxial electrospun fibers

To have an indication on the release of compounds from CA-PU fibers, CA-PU membranes were loaded with the fluorophore Rhodamine B (as a model compound) in core fiber of PU. Next, the membranes were dispersed in deionized water, as described in the methods section, and the concentration of Rhodamine B released as a function of time was measured. As Fig. 6 shows, about 50% of the Rhodamine B becomes released from the CA-PU membranes in the first three days, followed by a slower release in the following days, which should be attributed to the hydrophilic properties of CA. Note that such a release profile over about 10 days may be well suited for the generation of callus taking into account the plant callus cycle (Edelstein, 2004). In contrast, almost all Rhodamine B becomes released from CA membranes in the first three days. Such a fast release of compounds from the membranes could result in a too high concentration of the compound in the first days (possibly inhibiting callus growing) and too little release in the following days. CA is an insoluble and hydrophilic polymer and PU is an insoluble and hydrophobic. In the CA-PU coaxial electrospun fiber, the drug is loaded in the hydrophobic PU section, because the affinity with the water is relatively poor, it can slow down the precipitation of small molecule drugs. Besides, the release profiles of CA electrospun fiber exhibited a one-stage release characteristic. Moreover, because the drug is wrapped in the core of CA – PU coaxial electrospun fiber, the two-stage release kinetics is observed for CA-PU coaxial electrospun fiber, which proves that the location of the drug and the kinds of carrier material have a significant effect on the sustained release properties.

2.6. Toxicity of the membranes

The toxicity of CA-PU membranes, without 6-BA or loaded with 0.02%wt 6-BA, are evaluated by the somatic embryogenesis system of immature embryo derived from Liriodendron hybrid. The result is showed in Fig. 7 electrospun fiber membranes prepared in this experiment don’t have any effect on the callus uptake of plant cells (Fig. 7B and D). In addition, the callus on electrospun fiber membranes proliferate in much more fast larger size and darker color in comparison with the control plant with membrane free which proved that the proliferation rate is fast and in good condition. The auxin-added membrane is better than the absence one in more deep color, the capillary phenomenon could be attributed to the results as fiber membrane provokes enrichment and transportation of nutrients (Sarbati, Krishnaiah & Kamin, 2016) (Fig. 7E right). However, the traditional plastic membrane blocks transmission of nutrients (Fig. 7E left). This results demonstrate that the CA-PU fibers don’t induce any toxicity, but even promote callus proliferation which may a crucial of plant wound healing and plant grafting.
2.7. Wound healing potential of CA-PU membranes

Next we evaluated the potential of CA-PU membranes to promote the healing of wounds in plants. First, we made an incision (4 mm × 8 mm) in (about five months old) branches of Liriodendron using a lancet. Subsequently the incisions were wrapped with respectively a (traditional) plastic membrane, CA-PU membranes without auxins and membranes containing 0.02% or 0.2% of 6-BA. No bandaged incisions were used as control. The extent of wound healing was scored after ten days of healing. As showed in Fig. 8A (plant at the left side), without the use of membranes the wounds did not heal at all while the trunk is clearly visible. Using traditional plastic membranes (Fig. 8A (plant at the right side), the wounds became a little smaller, however, a trunk is still clearly visible in Fig. 8B-D one can clearly observe that the use of electrospun membranes clearly promotes plant wound healing. One reason could be the large specific surface area of electrospin which is beneficial for plant cell adhesion. A higher concentration of 6-BA did not induce a better wound healing (Fig. 8D). Note that this is consistent with the fact that high concentrations of auxins may inhibit plant growth. The wound healing pattern is shown in Fig. 8E-H, which helps us to understand the changing trend of healing process.
3. Conclusions

CA-PU coaxial electrospun fiber membranes were designed for the sustained release of exogenous auxins and had been fabricated successfully which is confirmed by FTIR and TEM. Importantly, the PU-core is completely wrapped by the CA-shell. The CA-PU fibers possess homogeneous and smooth surface, are well compatibility with water, show a high mechanical strength and high elastic modulus as well. We showed that such fibers are not toxic to plant cells and significantly improve both wound healing in plants and the survival rate of plant grafts. We conclude that CA-PU fiber membranes have value for plant wound healing and grafting.

Acknowledgements

National Natural Science Foundation of China (No.21644004), Jiangsu key lab of biomass-based energy and Materials (JSBEM2016011), Jiangsu specially-appointed professorship program (Sujiaoshi [2012]34), Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD), Top-notch Academic Programs Project of Jiangsu Higher Education Institutions (TAPP), Scientific Research Staring Foundation for the Returned Overseas Chinese Scholars, Ministry of Education of China and Jiangsu key lab of biomass-based green fuels and chemicals (JSBGC14001) and Natural Science Key Project of the Jiangsu Higher Education Institutions (16KJA220006) are acknowledged with gratitude. We also thank Advanced Analysis & Testing Center, Nanjing Forestry University for SEM characterization.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2017.04.020.