Applied Clay Science xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Applied Clay Science

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

Research paper

Nanotubes in nanofibers: Antibacterial multilayered polylactic acid/halloysite/gentamicin membranes for bone regeneration application

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ARTICLE INFO

Keywords:
Polylactic acid
Halloysite nanotubes
Electrospinning
Bone regeneration

ABSTRACT

A multilayered polylactic acid (PLA)/halloysite (HAL) porous membrane encapsulated with aminoglycoside antibiotic (gentamicin) was prepared by electrospinning technique. The prepared electrospun membrane displayed smooth morphology and uniform fiber distribution, while various layers of PLA mats were tightly bounded together. Significant enhancement in mechanical properties and stability was observed in membranes reinforced by HAL and the multilayered membrane loaded with gentamicin, while the drug loaded membrane showed no delamination. In addition, incorporation of HAL led to improvement in thermal stability of the PLA nanofibrous membranes. The in vitro drug release study of gentamicin loaded membrane initially shows a burst release of gentamicin, followed by slow rate of drug release up to 48 h. Antimicrobial efficacy of electrospun membrane was tested on a gram-positive (*Staphylococcus aureus*) and a gram-negative (*Escherichia coli*) bacteria, while results indicated that the gentamicin released from electrospun membrane has retained its antibacterial activity. These results signified the potential of multilayered porous PLA/HAL membranes to be utilized in prevention of infection in bone regeneration applications.

1. Introduction

The etiology of osteomyelitis (bone marrow inflammation) depends on the mechanism, it is mostly caused through the bloodstream or penetrating trauma while its eradication is known to be complex. The infection is typically caused by Gram-positive *Staphylococcus aureus* and Gram-negative Enterobacteriaceae and *Pseudomonas aeruginosa* (Reichert et al., 2006; Tsourvakas, 2012). Thus, local therapy by means of high concentration of an antibiotic, extended release with low concentration in the blood, is required to treat osteomyelitis and infected nonunion. The most commonly used antibiotics in local therapy are aminoglycosides e.g. gentamicin, β-lactam agents and quinolones. Gentamicin has a broad spectrum of activity against Gram-negative and Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* (Morawska-Chochół et al., 2014). Currently, the most applied commercial product containing gentamicin is Septopal – poly (methyl methacrylate) (PMMA) chain with 10 or 30 beads loaded with gentamicin. The large surface area of each bead (7 mm diameter and 0.2 g) leads to controlled and slow release of 7.5 mg of gentamicin sulphate, which is equivalent to 4.5 of mg gentamicin base. Gentamicin released by diffusion mechanism will reach at concentration of 400–600 μg on the first day, around 120 μg/day on the tenth day and 10 μg/day on the eightieth day of administration. Local therapy and minimal toxicity of Septopal has been confirmed by the results of pharmacokinetic studies, where the concentration of the antibiotic obtained in serum did not exceed 0.5 μg/mL (Reichert et al., 2006). However, the non-biodegradability of Septopal is considered to be a drawback since an additional surgery is required to remove the chain connected to the tissue. Nevertheless, implantation of Septopal is preferred by many orthopedic surgeons since the surgery site is well supplied with blood after its removal. (Reichert et al., 2006; Tsourvakas, 2012). Another widely used commercial product is resorbable collagen sponge with gentamicin, where antibiotic is suspended in sterilized bovine tendon. It is advised to utilize 1–3 sponges in patients weighing < 50 kg, and up to 5 sponges for patients above 50 kg. Gentamicin is released from the collagen carrier initially by diffusion, followed by enzymatic degradation of collagen which takes place within days up to a few weeks. The procedure involves treatment of the infected site with antibiotic-soaked

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https://doi.org/10.1016/j.clay.2017.12.016
Received 29 August 2017; Received in revised form 25 November 2017; Accepted 10 December 2017

cotton gauze. This method minimizes the risk of infections in various cases (e.g. in trauma patients), although additional supplementation of antibiotic is still needed (Reichert et al., 2006; Retzepi and Donos, 2010; Tsourvakas, 2012).

The local delivery system of antibiotics for treatment of bone and tissue infections and chronic osteomyelitis had been recognised as a commonly used method due to its ability to deliver high concentration of drugs without side effects or toxicity. The ideal antibiotic carrier in a local therapy should be able to deliver high dosage of drug at the infection site below the toxic level in comparison to traditional methods of antibiotic therapy (Gentile et al., 2011; Reichert et al., 2006; Tsourvakas, 2012). Halloysite nanotubes (HAL) were used as a drug container of gentamicin and reinforcing material for bone implants with poly (methyl methacrylate) bone cement (Wei et al., 2012), as well as with electrospun membranes with different polymers, improving the physical and mechanical properties of the polymer matrix (Lvov and Abdullayev, 2013). HAL are naturally formed tubular pieces of 1:1 clay, with positively charged octahedral (Al-OH) lumen and negatively charged external tetrahedral (Si—O) surface with water in the interlayer and at the end of the surface. Destruction of crystal structure of halloysite begin at 400 °C, while the tubular structure is retained after heating and drying till 900 °C (Yuan et al., 2012). In addition, halloysite may also occur in platy, spherical, or partly rolled form, while the tubular structure is the most interesting form utilized in numerous applications in various fields starting from traditional ceramics to advance medical applications (Churchman et al., 2016; Pasbakhsh et al., 2013). Moreover, halloysite presents descent dispersion in polymers with low agglomerating content in comparison to typically used clays like montmorillonite or kaolinite. Differing chemistry between the surface and lumen of HAL facilitate its incorporation in polymer matrix and provide unique properties by altering the pattern of the polymer (Yuan et al., 2015).

Vergaro et al. (2010) studied interaction of HAL with different cells like human epithelial adenocarcinoma cell line (HeLa) and human breast cancer cell line (MCF-7) and carried out cytotoxicity tests with MMT and Trypan blue, both for untreated HAL and HAL functionalized with APTES (HAL Dragonite from Applied Minerals Inc.). Results indicated that in biological conditions, HAL might be highly hydrated by wetting the surface which may result in a higher level of biocompatibility. Furthermore, slight adhesion of human dermal fibroblasts, faster compared to silica and montmorillonite were observed. Study revealed that halloysite is nontoxic and relatively less harmful in comparison to NaCl. This was further confirmed when addition of 75 μg/mL of halloysite in cell culture did not eradicate the cells, while incorporation of 50 μg/mL of NaCl led to cell death. It was proved that the maximum safe concentration of halloysite is 0.1 mg/mL and thanks to submicrometer dimensions it might be removed by macrophages (Vergaro et al., 2010). In recent years, number of studies have been done on fabrication of electrospun HAL/polymer composites for drug delivery applications of variety of drugs (Patel et al., 2015). Xue et al. (2015) developed electrospun microfiber membranes embedded with metronidazole-loaded HAL for guided tissue regeneration, while results indicated sustained drug release. In another study, dual drug delivery of both hydrophilic and hydrophobic drugs have been performed by poly (L-lactide)/halloysite nanotube electrospun mats (Zhang et al., 2015) Recently, Tohidi et al. (2016) have prepared poly(lactic-*co*-glycolic acid)/chitosan electrospun membrane containing amoxicillin-loaded halloysite nanotubes. Results indicated high potential of HAL to be utilized as a nanocarrier for controlled drug release. Similar results were reported by Makaremi et al. (2017) on encapsulation of various types of HAL.

The aim of this project was to produce a cytocompatible, nontoxic and mechanically stable multilayered porous membrane encapsulated with aminoglycoside antibiotic (i.e. gentamicin) for bone regeneration with a high concentration of drug at the first stage of release and extended drug release. Owing to its high biocompatibility and rising interest, HAL were used as a reinforcing material. Fabricated membrane consists of 3 different layers, while the thickness of each layer is around 30–40 μm. The first layer is porous and soaked with an antibiotic, while the second layer contains porous fibers loaded with halloysite nanotubes in pores and adsorbed antibiotic. The third layer comprise of 4% halloysite nanotubes within non-porous and smooth polylactic acid (PLA) nanofibers and were soaked in gentamicin solution. Un-soaked layers were prepared and investigated in the experiment as well. It is known that PLA may not be the best candidate for a biodegradation in the body, as the membrane is expected to be degraded after 1–2 months in the body. However, the acquired knowledge aimed at achieving deeper comprehension regarding the properties of PLA/HAL multilayered biodegradable electrospun membrane relevant for the design of new hybrid sustainable materials, particularly for wound dressing applications.

2. Experimental

2.1. Materials

PLA (grade 3051D) was purchased from Nature-Works, USA. Three solvents were chosen to prepare solutions: *N, N*-dimethylformamide (DMF) (anhydrous, 99.8% from Sigma-Aldrich), Chloroform (CF) (Sigma-Aldrich) and Dichloromethane (DCM) (Sigma-Aldrich). Halloysite nanotubes (HAL) Dragonite-HP untreated (length 50–1500 nm and surface area − 65 m^2/g) (Pasbakhsh et al., 2013) were used to reinforce the membranes. Garamycin (Gentamicin Sulphate ampules for injection) with concentration of 80 mg/2 mL was used as a drug adsorbed to HAL and PLA fibers.

2.2. Preparation of single and multi-layer membranes

Table 1 summerizes the details of all prepared membranes in this study. The polymer solution was prepared by dissolving PLA in a combination of two solvents in 3:1 ratio chloroform (CF) and dichloromethane (DCM) for porous fibers or 3:1 CF and dimethylformamide (DMF) for smooth fibers. PLA concentration of each solution was 14% (w/w). Layer 1 with pores was prepared from a solution of PLA/

Table 1
Description of preparation of single and multi-layer membranes.

Name of the layer	Description	Materials
Layer 1	Porous electrospun PLA without HAL	PLA/CF + DCM
Layer 2	Porous electrospun PLA with HAL in pores of the fibers	PLA/CF + DCM/HAL
Layer 3	Smooth electrospun PLA with HAL inside fibers	PLA/CF + DMF/HAL
Complete set 1 (CS1)	All above layers in one sheet	PLA/CF + DCM, PLA/CF + DCM/HAL, PLA/CF + DMF/HAL
Layer 1S	Porous electrospun PLA without HAL soaked with GM	PLA/CF + DCM/GM
Layer 2S	Porous electrospun PLA with HAL in pores of the fibers, soaked with GM	PLA/CF + DCM/HAL/GM
Layer 3S	Smooth electrospun PLA with HAL inside of the fibers, soaked with GM	PLA/CF + DMF/HAL/GM
Complete set 2S (CS2S)	All above layers in one sheet	PLA/CF + DCM/GM PLA/CF + DCM/HAL/GM PLA/CF + DMF/HAL/GM