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Improving the degradation behavior and *in vitro* biological property of nano-hydroxyapatite surface- grafted with the assist of citric acid

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ABSTRACT

To obtain ideal nano-hydroxyapatite(n-HA) filler for poly(lactide-co-glycolide) (PLGA), a new surface-grafting with the assist of citric acid for nano-hydroxyapatite (n-HA) was designed, and the effect of n-HA surface-grafted with or without citric acid on *in vitro* degradation behavior and cells viability was studied by the experiments of soaking in simulated body fluid (SBF) and incubating with human osteoblast-like cells (MG-63). The change of pH value, tensile strength reduction, the surface deposits, cells attachment and proliferation of samples during the soaking and incubation were investigated by means of pH meter, electromechanical universal tester, scanning electron microscope (SEM) coupled with energy-dispersive spectro-scopy (EDS), fluorescence microscope and MTT method. The results showed that the introduction of citric acid not only delayed the strength reduction during the degradation by inhibiting the detachment of n-HA from PLGA, but also endowed it better cell attachment and proliferation, suggesting that the n-HA surface-grafted with the assist of citric acid was an important bioactive ceramic fillers for PLGA used as bone materials.

1. Introduction

Nano-hydroxyapatite(n-HA) is a major inorganic component of natural bone, and it is one of important bioactive ceramic fillers for many polymers [1–3], for example, poly(lactic acid-co-glycolic acid) (PLGA), a copolymer of poly lactic acid (PLA) and polyglycolic acid (PGA). Therefore, many researchers are concentrating on fabricating n-HA/PLGA composite by combination of the individual advantages of n-HA and PLGA [4–6]. However, the unmodified n-HA or undesirable modified n-HA would be unfavourable for improving the bend strength of PLGA, whose bend strength decreased nearly 17% than that of PLGA because of the poor interface adhesion, when the n-HA content was over 15 wt% [7–9]. Therefore, increasing attention has been focused on exploring new surface-modification methods for n-HA, so as to obtain the n-HA/PLGA composite with higher original mechanical property than PLGA. In our previous works, we studied three new combination grafting methods for n-HA, which was carried out with the assist of stearic acid, silane coupling reagent (KH550) and L-lysine, respectively, and we concluded that the surface-modified n-HA above could increase the bend strength and tensile strength of PLGA to some extent. Especially, our recent study results indicated that the surface-grafting with the assist of citric acid for n-HA brought about more excellent mechanical enhancement effect for PLGA, due to the better interface adhesion between n-HA and PLGA, compared with the other surface-grafting methods. For example, the bending strength and tensile strength were both over 20% higher than those of pure PLGA, when 3 wt% n-HA was added. Moreover, it was still enhanced 8% and 6% than those of pure PLGA, respectively, even 15 wt% n-HA was introduced into PLGA. So the surface-grafting with the assist of citric acid for n-HA was regarded as a new ideal surface-modification method [10–12].

However, for absorbable materials, *in vitro* degradation rate could be helpful to predict the degradation rate *in vivo* according to the *in vitro* degradation result, and undesirable degradation rate might directly affect the bone healing procession, so it is one of crucial factors to determinate the feasibility to be used as bone materials [13,14]. Li HY, et al. reported that the incorporation of HA

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into PLGA accelerated the degradation rate of PLGA based on the soaking result in phosphate buffered saline (PBS) for 8 weeks [15]. Similarly, we also studied the degradation behavior of n-HA/PLGA composite with n-HA surface-grafted by combination stearic acid, and the results indicated that the modified n-HA greatly accelerated the degradation of PLGA, whose bend strength decreased nearly 50%, while the bend strength of pure PLGA decayed less than 10% after being soaked for 6 months [16]. Therefore, considering the practical bone healing time and the degradation difference between *in vivo* and *in vitro* [17], it is necessary to explore the other surface-modified n-HA, which is expected to decrease a little for the degradation rate of PLGA. Nevertheless, for the n-HA surface-modified with the assist of citric acid, as we know, citric acid displays acidity in water because of molecular structure of the carboxyl groups, so it is unclear whether the incorporation of citric acid into n-HA might further accelerate the hydrolytic degradation of PLGA, which would affect the practical application. Meanwhile, the influence of the introduction of citric acid into n-HA on the bioactivity in simulated body fluid (SBF) is also expected to be investigated, which is helpful to predict the bone-bond ability *in vivo*. Therefore, it is essential to investigate the degradation behavior of n-HA/PLGA composite by soaking it in SBF.

Additionally, as we know, bone materials should be non-toxic and have good biocompatibility. Moreover, it is reported that the local acidic environment is adverse for cells in a living body, which would result in poor biocompatibility and inflammatory, even it might interfere directly the bone healing *in vivo* [18]. Generally speaking, for a new implant materials, the cell culture experiment *in vitro* is often believed to be a popular and simply initial evaluation ways for assessing the biocompatibility, based on the result of cell attachment and proliferation on the surface of materials, which is an important criterion to preliminarily decide the feasibility of implantation in body [19]. Therefore, whether the new ideal surface-modified n-HA would be toxic, especially, whether the introduction of citric acid would produce a poor biocompatibility, which is expected to be studied by the cell culture experiment.

Based on these consideration and our previous study results, being a successive work, here, we are intended to study systematically *in vitro* degradation behavior and the cells viability of the n-HA/PLGA composite, including the change of pH value, tensile strength reduction, the surface deposits, cells attachment and proliferation of samples during the soaking and cells culturing time, comparing with the corresponding n-HA surface-grafted without citric acid and pure PLGA. The main aim of the study is to investigate comprehensively whether the introduction of citric acid into n-HA would accelerate the hydrolytic degradation and bring about negative effect on biocompatibility of n-HA/PLGA composite, which would be expected to provide significant reference for preparing n-HA/PLGA composite used as bone materials in future.

2. Materials and methods

2.1. Surface-modification of n-HA

Surface-grafted n-HA with or without citric acid (noted as g2-n-HA and g1-n-HA) were both prepared in our laboratory [12]. In brief, 10 g n-HA and 10 g citric acid was suspended in 500 ml N, N dimethylformamide (DMF) with stirring at 150 °C for 11 h under nitrogen, then washed by ethanol and vacuum-dried. Secondly, 8 g powder was surface-grafted by 8 g D, L-lactide (which was prepared in our laboratory) with 0.004 g stannous octanoate ($Sn(Oct)_2$) (which was purchased from Aldrich (Milwaukee, USA)) at 140 °C for 20 h under nitrogen. The sample was obtained and noted as g2-n-HA, after being washed with excessive dichloromethane and vaccum-dried. The n-HA surface-grafted without citric acid was prepared in the same procedure, and it was noted as g1-n-HA. The size of the two particles was about 100–120 nm in length and 20–40 nm in width. All other agents were analytical grade.

2.2. Preparation of g-n-HA/PLGA composites

The four composites were prepared by solution mixing method according to our previous report [12]. First, the pure PLGA was dissolved in dichloromethane, and pre-calculated g2-n-HA or g1-n-HA, uniformly dispersed in mixture solution of ethanol and dichloromethane, was slowly added into PLGA solution with the addition of 3 wt% and 25 wt%, respectively. Then the mixtures were stirred with ultrasonic treatment for more than 4 h, and the precipitates were washed with excessive ethanol and dried under vacuum at 40 °C for 48 h, which was noted as g2-n-HA/PLGA composites and g1-n-HA/PLGA composites, respectively. Finally, the two types of g-n-HA/PLGA composites and pure PLGA were pressed into specimens with dimensions of 50 mm × 6 mm × 0.5 mm, and the samples were annealed at 110 °C for 30 min in a vacuum oven.

2.3. In vitro *soaking*

The SBF, whose ion concentration was similar to that of human blood plasma, was prepared by dissolving reagent-grade NaCl, $NaHCO_3$, KCl, $K_2HPO_4{\cdot}3H_2O$, $MgCl_2{\cdot}6H_2O$, $CaCl_2$, Na_2SO_4 and tri-(hydroxymethyl) aminomethane ($(CH_2OH)_3CNH_2$) in deionized water, and pH was adjusted to 7.40 at 37 °C with 0.1 mol/l HCl [20]. The specimens of g-n-HA/PLGA composites and pure PLGA with dimensions of 50 mm × 6 mm × 0.5 mm were immersed in inert plastic tubes containing 20 ml of SBF for 2,4 and 8 weeks in a rocking water bath at 37 °C. The specimens were taken out from SBF after being soaked for different time, rinsed gently with deionized water several times, dried completely in a vacuum oven at 40 °C, absorbed with filter paper prior to dry.

The specimens of the two types of g-n-HA/PLGA composites and pure PLGA were both tested before and after being soaked, respectively, including the change of pH value, tensile strength reduction and surface morphology. The pH value was measured by pH meter. The tensile strengths of specimens were measured according to GB/T1042-92 at a speed of 2 mm/min, at 25 °C and a relative humidity of 60%. The surface deposits of samples were observed by scanning electron microscope (SEM) (KYKY-2800 KYKY, China), and the elements of surface deposits were analyzed by energy-dispersive spectro-scopy (EDS), after being uniformly sputtered with a gold layer.

2.4. In vitro *cell experiment*

Human Osteosarcoma Cell Line MG-63 was bought from West China Hospital of Stomatology Sichuan University, which was used to preliminarily evaluate *in vitro* cells viability. The samples of the two types of g-n-HA/PLGA composites and pure PLGA with 6.0 mm in diameter and 0.2 mm in thickness were sterilized with ethylene oxide gas and placed in a 96-well cell culture plate. Approximately 2×10^4 cells of MG63 were seeded on the samples, following undisturbed in an incubator for 3 h, then added an additional 1.0 ml of culture medium into each well, and the samples/cell were cultured in an incubator (37 °C with a humidified 5% CO_2 atmosphere) for 3 days. Empty wells without sample were treated in the same procedure as controls.

After incubation for 1, 2 and 3 days, samples were taken out and rinsed with PBS two times, and dyed for 20 min by dropping two drops of acridine orange solution with 0.1 $mg\,mL^{-1}$onto sample, then rinsed with PBS two times again, and Fluorescence Microscope (Olympus B × 60) was used to observe the morphology and growth of cell.