Fabrication of biocomposites composed of natural rubber latex and bone tissue derived from MC3T3-E1 mouse preosteoblastic cells

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Abstract Natural rubber latex (NRL) is mainly used around traditional industrial products, but currently their target application is continuously expanding into tissue engineering. To broaden our knowledge of application in tissue engineering of NRL, we have focused on the surface modification of NRL nanoparticles through the biomineralization of hydroxyapatite (HA) using simulated body fluid in order to create a better cytocompatibility with controlled cell adhesion and mineralization properties in osteogenic culture to determine the effect on bone outcomes. Using MC3T3-E1 mouse osteoblastic-like cells incubated with NRL nanoparticles coated with HA layer, we have examined the osteogenic differentiation and expressions of multiple proteins and characteristic genes of mature osteoblast. We have successfully prepared the biocomposites composed of NRL and bone tissue.

Keywords Natural rubber latex, MC3T3-E1 cells, Biocomposites, Osteogenic differentiation

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Introduction

Natural rubber latex (NRL) is a type of high molecular weight polymeric substance. Currently, more than 90% of NRL comes from one single tropical tree species – Hevea brasiliensis (para rubber tree). NRL is mainly used around traditional industrial products, such as gloves, condoms, balloons, medical equipment, and tires, but currently their target application is continuously expanding into tissue engineering. Recently, promising results in the use of NRL from Hevea brasiliensis rubber tree to produce replacement and regeneration tissues have been reported.1-3 Among them, wound healing in cutaneous tissues, eardrum replacement, bone regeneration, and dental alveolus replacement afford a unique means to repair or replace the failing organs or tissues.4 The primary particle diameter of NRL is in the range of 100–300 nm, which is promising for nanoparticles used in biotechnology including pharmaceutics, tissue engineering, and regenerative medicine. The nanoparticles are usually taken up through endocytosis. The particle size between 20 and 200 nm is suitable for drug delivery applications based on in vivo studies.4

In our preliminary study, we have demonstrated the latex cytocompatibility in vitro against cultured human lung carcinoma (A549) and mouse calvaria preosteoblast (MC3T3-E1) cells. The NRL nanoparticles enhance MC3T3-E1 cell proliferation and viability at concentrations of 10.0 μg/mL in comparison with that of the control. In contrast, the NRL particles are found to be toxic to the A549 cells (cell viabilities are below 60% at concentration of 100 μg/mL). The administration of NRL particles could effectively inhibit the proliferation of A549 cells within 1 day. The estimated values of half maximal inhibitory concentration (IC50) are 330 ± 20 μg/mL for A549 cells and 3.97 ± 0.10 mg/mL for MC3T3-E1 cells in the range of 10 μg/mL–10 mg/mL (Figure S1, Supplementary information). Our data showed that the addition of even as much as 1.0 mg/mL of NRL nanoparticles in cell culture did not kill tested MC3T3-E1 cells. The NRL nanoparticles

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exposure to biological tissue is expected when one applies NRL for anticancer drug delivery.

In order for NRL nanoparticles to be employed in regenerative medicine, they need to have designed biological interaction with cells as well as favorable mechanical properties. Cells can delicately sense and respond to external nanoscale features in intricate living systems. With this in mind, an in-depth understanding of cell differentiation may bring a new perspective to regenerative medicine including new knowledge of the time-dependent intensity. Characterization of the morphology was observed through field emission scanning electron microscope (FE-SEM: SU6600, Hitachi Ltd.). The operated accelerating voltage was 15 kV and the specimens were coated with a thin layer of gold and palladium (Au/Pd 6:4) with a thickness of ~20 nm on copper grids with 200 mesh size. Fourier transform infrared (FTIR) spectroscopic imaging measurements were performed using a Perkin-Elmer Spectrum Spotlight 400 Microscope System. This system is equipped with a liquid N₂ cooled Mercury-Cadmium-Telluride MCT detector. To construct FTIR maps, spectra were collected in continuous scan mode for sample area of 200 x 200 μm² with a resolution of 1.65 μm/pixel by one scan for each spectrum of the specimens under the Ge attenuated total reflectance (ATR) crystal. Spectra were collected from 4000 to 680 cm⁻¹ with a resolution of 8 cm⁻¹ and integrated by taking the areas under the curve between the limits of the peaks of interest. Data acquisition was carried out by means of the Spotlight software package. The thermal behavior was determined by thermo-gravimetric/differential thermal analysis (TGA&DSC, TA Instruments) in the temperature range of 20–900 °C at a heating rate of 5 °C/min under dry air. Sample weight of NRL-HA was normalized at 2.8 mg. The NRL nanoparticles before biomineralization was used for a control.

In vitro cell culture and cell viability

MC3T3-E1 mouse calvaria preosteoblastic cells obtained from ATCC were cultured in Alpha Minimum Essential Medium (a-MEM) (Gibco®, Life Technologies) at pH 7.4 supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco®, Life Technologies) and 1% antibiotic-antimycotic mixture (Nacalai-Tesque). Upon reaching confluence, cells were cultured on 10 cm dishes in an atmosphere of 5% CO₂ and 95% relative humidity at 37 °C. MC3T3-E1 cells at 2–4 passages were used in the experiment.

Human lung carcinoma A549 cells (ATCC) was used as a reference cell and cultured in RPMI-1640 (Wako Pure Chemical Industries) supplemented with 10% FBS including 1% antibiotic-antimycotic mixture. A549 cells at 2–5 passages were used in the experiment.

The cells were seeded in 96-well plates at a cell concentration of 2 x 10⁴ cells/cm² in 100 μL of medium and cultured for 24 h. The NRL-HA and/or NRL suspensions at different concentrations (from 12 μg/mL to 1.2 mg/mL) were diluted with complete culture medium and added to each well. After incubation for 24 h in an atmosphere of 5% CO₂ and 95% relative humidity at 37 °C, the cell viability was assessed by WST-8 assay (Dojindo) according to the manufacturer’s instructions. The WST-8 colorimetric test is measuring the activity of intracellular dehydrogenase activity, which is proportional to living cells. The optical density was read using a Multiskan FC (Thermo Fisher Scientific) at 450 nm for the absorbance and at 650 nm for the subtract background absorbance, respectively.

Differentiation

For osteogenesis, MC3T3-E1 were seeded at a density of 4 x 10⁴ cells/cm² in 200 μL of osteogenic medium and cultured with...
NRL-HA and/or NRL nanoparticles, as reference. The effect of NRL-HA on cell differentiation was determined for 20 days in osteogenic medium, consisting of α-MEM, 10% FBS, 1% (v/v) ascorbic acid (TaKaRa), 0.2% (v/v) Hydrocortisone (TaKaRa), and 1% antibiotic-antimycotic mixture. The medium was changed with a fresh one every 3 days, 6 times during the 20 days incubation.

For the calcium deposition, after 20 days, the osteoinduction of the MC3T3-E1 cells in osteogenic culture was analyzed by measuring the absorption of treated cells by Alizaline Red staining (PG Research) according to the manufacturer’s instructions. The optical density at 450 and 650 nm, for the subtract background absorbance, were read using a microplate spectrophotometer (Multiskan FC, Thermo Fisher Scientific).

**Gene expression**

The total RNA was extracted from cells, induced to osteogenic differentiation for 3, 10 and 20 days, using RNeasy Mini Kit (Qiagen) with a residual genomic DNA Eraser following the protocol. Then the RNA was subjected to reverse transcription using a SuperScript® III (Invitrogen, Life Technologies) following the manufacturer’s instructions. The resulting complementary DNA (cDNA) yield was then subjected to real-time polymerase chain reaction (RT-PCR) using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The results were analyzed with Sequence Detector software (Applied Biosystems). Reaction solutions included SYBR Green Mastermix, 1 μM forward and reverse predesigned primers (Table 1), and 10 μL cDNA template in a 30 μL volume. The cDNA samples were analyzed for expression of Runx2, Sp7/Osterix, alkaline phosphatase (Alp), and osteocalcin (Bglap), relative to the β-actin (ACTB) as an internal standard for sample normalization.

**Statistics**

Statistical analysis was performed using Student’s t-test and one-way analysis of variance with Dunnett’s post hoc testing, and significance was considered at a probability of $p < 0.05$.

**Results and discussion**

**Characterization of NRL-HA nanoparticles**

The main reason for the formation of NRL-HA nanoparticles is due to the electrostatic interaction between negatively charged layer along the NRL surface and Ca$^{2+}$, which deposited calcium ions, in turn, interacted with phosphate ions (PO$_4$)$^{3-}$ in the SBF. The HA then grew spontaneously accompanied by consuming the calcium and phosphate ions to form apatite.$^{5,9}$

The presence of layers can be demonstrated by monitoring the zeta potential, which change from negative toward positive direction when HA is at the outside and NRL (~70 mV) is at the inside as shown in Figure 1(a). The zeta potentials of the NRL-HA nanoparticles at pH 7.4 exhibit the negative values ($-40$ mV), indicating somewhat dense HA layers including cations, (e.g. Ca$^{2+}$ and CaOH$^+$) on the NRL particle surfaces. The values increase continuously with decreasing pH to attain 0 mV at pH 2.0.

For comparison, the zeta potential of precipitated HA without NRL particles is shown (0.01 wt.%). Results are expressed as mean ± S.D. (Standard deviation) ($n = 5$). (b) Particle size distribution with PDI of NRL and NRL-HA after biomineralization at concentration of 0.01 wt.% at pH 7.4.
The particle size of NRL-HA nanoparticles from DLS measurement is presented in Figure 1(b). At pH 7.4, the average particle size of pristine NRL is around 120 nm with polydispersity index (PDI) of 0.375 at concentration of 0.01 wt.%. The average size of the NRL-HA nanoparticles is 200 nm having narrower PDI of 0.164 in comparison with that of NRL nanoparticles before coating with HA layer.

Figure 2 shows the morphological feature of NRL-HA nanoparticle as revealed by FE-SEM image. The nanoparticle exhibits rather coarse surface having a size of ~1 μm (Figure 2(a)). In addition, small discrete particle after biomineralization is also observed in which the particle size is ~200 nm (Figure 2(b)). We can postulate that the negatively charged layer on the NRL surface act as nucleating sites and HA crystal growth front as well. However, the top surface covered with HA layer may show less-ordered crystal structure (low crystallinity) as reported by our previous study of DNA mineralization in SBF.8

The TGA curve, derivative analysis (DTG) and DTA of the NRL-HA nanoparticles and the pristine NRL are shown in Figure 3. The NRL nanoparticles lost ~3% mass in the temperature range 120–200 °C (Figure 3(a)). A significant mass loss (~93%) from 200 to 510 °C is observed, accompanied by the four corresponding exothermic peaks at 240, 301, 404, and 453 °C, which are due to the pyrolysis and/or decomposition of NRL (Figure 3(b)).

In contrast, NRL-HA nanoparticles indicate the weight loss of ~80% after freeze-drying in the temperature range from 200 to 510 °C (Figure 3(c)). The NRL in the NRL-HA decomposes in two stages, with a weight loss of 45% between 200 and 400 °C and a weight loss of 35% from 400 up to 510 °C, accompanied with the several peaks in DTG (252, 320, 362, 407, and 494 °C). At the same time, we can find that the two exothermic peak temperatures (i.e. 429 and 494 °C) of the NRL degradation in the NRL-HA nanoparticles are shifted to a higher temperature as compared with that of the pristine NRL (i.e. 404 and 453 °C) (cf. DTA curves: Figure 3(b), (d)).

The excellent thermal stability of HA beyond the temperature 1000 °C is well known in the literature.13 The thermal stability of the incorporated NRL in NRL-HA nanoparticles is much better than that of the pristine NRL, despite the low crystallinity of HA. The calculated weight ratio of NRL/HA in the NRL-HA nanoparticles is \(\text{ca}}.84/16\) by taking the combustion residue of the pristine NRL into account.

FTIR image is generated by plotting the principal components analysis (PCA) to illustrate the relative distribution of the components (Figure 4). The NRL-HA nanoparticles are divided into five components as revealed by PCA. In all components, original peaks of both NRL and HA are assigned. The sensitive bands at 2944 (asymmetric stretching: \(v_\text{s}(\text{CH}_2)\)), 2852 (symmetric stretching: \(v_\text{as}(\text{CH}_2)\)), 836 cm\(^{-1}\) (cis\(\text{CH}==\text{CH}\)) are attributed...
to cis-1,4-polyisoprene. The sensitive bands at 1628 (peptide: υ(C=O)), 1552, 1238 (peptide: υ(C=O/N–H)), and 1296 cm⁻¹ (asymmetric stretching mode: υ as(PO2–)) are attributed to the surface protein of NRL. The sensitive band at 1036 cm⁻¹ (υ3(PO4³–)) is attributed to HA.¹⁴ The bands at 1460 cm⁻¹ (υ(CO3²–)) and 1388 cm⁻¹ (υ(CO3²–)) are presented.¹⁵,¹⁶ That is, the phosphate groups or hydroxyl groups in HA crystallites have replaced the carbonate acid in SBF accompanied with the formation of carbonate hydroxyapatite, which indicate low crystallinity by incorporation of carbonate acid into HA via an anion exchange reaction. A band located at around 3200 cm⁻¹ was assigned to the structural OH stretching mode (υ(OH)) from both NRL and HA. In the panels (2) and (5) in Figure 4, HA-rich compositions are detected in NRL particles. Although the information of them should be considered in more detail, the NRL-HA nanoparticles with different compositions are successfully prepared in the SBF. The constituents of the NRL-HA nanoparticles are mixture at present as seen in PCA. However, the cytocompatibility analysis has not been reported. The cytotoxicity of the NRL-HA nanoparticles were measured against MC3T3-E1 and cancer cells.

**Cytotoxicity of NRL-HA nanoparticles**

The viability of MC3T3-E1 cells is very sensitive to the NRL nanoparticle concentration. Beyond concentration of 10.0 μg/mL, NRL nanoparticles enhance MC3T3-E1 cells and viability in the range of 130–150% in comparison with that of control (Figure 5(a)). On the other hand, MC3T3-E1 cells exhibit no toxic effects and are not sensitive to NRL-HA nanoparticles, accompanied with high metabolic and dehydrogenase activity of the cells at concentration up to 1.2 mg/mL (Figure 5(a)).

![Figure 4 FTIR image of NRL-HA nanoparticles after biomineralization obtained from the selected 200 × 200 μm² area (left panel). FTIR spectra of corresponding five constituents (a)–(e) by plotting PCA (right panel) in the region of 4000–680 cm⁻¹](image)

![Figure 5 Cell viability as measured by WST-8 assay using (a) MC3T3-E1 and (b) A549 cells after 24 h of incubation with NRL and/or NRL-HA nanoparticles of different concentrations. Data were expressed as mean ± S.D. (n = 5)](image)

Note: * indicates p < 0.05 compared with control.
As seen in Figure S1 (Supplementary information), the administration of NRL particles could effectively inhibit the proliferation of A549 cells within 1 day. The estimated IC\textsubscript{50} values are 330 ± 20 μg/mL for A549 cells in the range of 10 μg/mL to 10 mg/mL. In contrast, the NRL-HA nanoparticles are found to be no toxic to the A549 cells (cell viabilities remain constant up to 1.2 mg/mL) (Figure S1(b)). For HA layer on the surface of NRL particle, it is possible to enhance the cell viability even at high concentration dose of up to 1.2 mg/mL. These results indicate that the HA coating on NRL nanoparticles causes significant changes in the cytotoxicity of both cells.

**Gene expression and matrix mineralization of MC3T3-E1 cells**

The gene expression of Runx2, Sp7/Osterix, alkaline phosphatase (Alp), and osteocalcin (Bglap) relative to housekeeping gene (ACTB) were determined by RT-PCR at day 3, day 10, and day 20. Both NRL and NRL-HA nanoparticles show relatively stable expression of Runx2 from 3 days to 20 days (Figure 6(a)). The MC3T3-E1 cells in mineralized matrices exhibit higher expression of Runx2 for the late stage of osteogenic differentiation (at day 20). But there are no statistical difference between any of samples and control throughout 20 days of culture. The Sp7/Osterix is also considered as a marker for the early stage osteogenic differentiation.\textsuperscript{17} The expression of Sp7/Osterix shows same trend with Runx2 (Figure 6(b)). The expression of Alp gene is elevated up to day 20. Since Alp is generally considered as an early marker of osteoblast phenotype and calcified matrix production.\textsuperscript{17} The NRL-HA nanoparticles promote the Alp gene expression than control at day 20 (Figure 6(c)), indicating the up-regulation leads to matrix maturation.

This promotion effect is further confirmed by the expression of the Bglap gene, which is a typical marker for the late stage of osteogenic differentiation. The cells cultured with NRL-HA nanoparticles exhibit significantly higher expression of Bglap in 10 days in comparison with that of NRL nanoparticles (Figure 6(d)).

Interestingly, for osteogenic differentiation of MC3T3-E1 cells, there are no statistical difference in osteogenic differentiation between control and loading of NRL nanoparticles of 0.1 mg/mL throughout 20 days of culture, as shown in Figure 7.

To examine matrix mineralization, 20 days cultures were fixed and stain by Alizarine solution, which stains calcium deposits as another marker of osteogenic differentiation. Interestingly, for osteogenic differentiation of MC3T3-E1 cells, there are no statistical difference in absorbance between any of samples except for NRL particles with 0.1 mg/mL, in which
nanoparticles caused significant changes in the cytotoxicity of both MC3T3-E1 and A549 cells. The NRL-HA nanoparticles promoted the Alp gene expression than control at day 20. After 20 days of incubation with osteogenic supplement, both rather stable gene expression and calcium deposition were observed from MC3T3-E1 cells cultured with both NRL and NRL-HA nanoparticles. We have successfully prepared the biocomposites composed of NRL-HA and bone tissue. The results showed promise of the NRL nanoparticles for application in bone tissue engineering. Further efforts are needed to explore the mechanical properties of the biocomposites and long-term toxicity in pre-clinical setting.

Conclusions

We have demonstrated the surface modification of NRL nanoparticles through the biomineralization of HA layer using SBF at 36.5 °C. The formation of NRL-HA nanoparticles were characterized by monitoring the zeta potential with different pH environment, morphological observation, DLS measurement, and FTIR imaging technique. The HA coating on NRL nanoparticles caused significant changes in the cytotoxicity of both MC3T3-E1 and A549 cells. The NRL-HA nanoparticles promoted the Alp gene expression than control at day 20. After 20 days of incubation with osteogenic supplement, both rather stable gene expression and calcium deposition were observed from MC3T3-E1 cells cultured with both NRL and NRL-HA nanoparticles. We have successfully prepared the biocomposites composed of NRL-HA and bone tissue. The results showed promise of the NRL nanoparticles for application in bone tissue engineering. Further efforts are needed to explore the mechanical properties of the biocomposites and long-term toxicity in pre-clinical setting.

Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.
Supplementary data
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