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Bioactivity and circulation time of PEGylated NELL-1 in mice and the potential for osteoporosis therapy

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Abstract

Osteoporosis is a progressive bone disease due to low osteoblast activity and/or high osteoclast activity. NELL-1 is a potential therapy for osteoporosis because it specifically increases osteoblast differentiation. However, similar to other protein drugs, the bioavailability of NELL-1 may be limited by its *in vivo* half-life and rapid clearance from body. The purpose of the present study is to prolong NELL-1 circulation time *in vivo* by PEGylation with three monomeric PEG sizes (5, 20, 40 kDa). While linear PEG 5k yielded the most efficient PEGylation and the most thermally stable conjugate, linear PEG 20k resulted in the conjugate with the highest Mw and longest *in vivo* circulation. Compared to non-modified NELL-1, all three PEGylated conjugates showed enhanced thermal stability and each prolonged the *in vivo* circulation time significantly. Furthermore, PEGylated NELL-1 retained its osteoblastic activity without any appreciable cytotoxicity. These findings motivate further studies to evaluate the efficacy of PEGylated NELL-1 on the prevention and treatment of osteoporosis.

Keywords

NELL-1; PEGylation; Characterization; Bioactivity; Circulation time

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Conflict of interest

Drs. Wu, Ting, Soo, and Zhang are co-founders of Bone Biologics, Inc., which sublicenses NELL-1 related patents from the UC Regents.

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1. Introduction

NEL-like molecule-1 (NELL-1) protein is widely studied in bone regeneration as an osteogenic growth factor with higher specificity to osteoblast cells compared to the growth factors currently used such as BMP-2 [1–4]. NELL-1 is a secreted homotrimer protein with molecular weight up to 400 KDa. The subunit of NELL-1 contains 810 amino acids and a molecular weight of about 90 KDa before N-glycosylation and oligomerisation [5]. Previous studies suggested that NELL-1 can specifically modulate the osteochondral lineage and induce bone formation in various kinds of animal models from rodents to sheep [1, 6]. Recently, Kwak et. al have demonstrated that the locally intramedullary application of NELL-1 in the femurs of ovariectomy (OVX)-induced osteoporotic female rats could enhance rat bone quality and prevent osteoporosis [7]. *In vivo* studies further indicated that the deficit of Nell-1 gene or loss NELL-1 function may contribute to the development of osteoporosis in animal and clinical researches [8, 9]. These studies suggest that the NELL-1 protein has potential to be used for treatment of osteoporosis by simple intravenous injection.

NELL-1 is often applied in local tissues (spine, femur, calvaria, etc) by being loaded onto various carriers including tricalcium phosphate (TCP) particles [10], demineralized bone matrix (DBM), and PLGA scaffold [2, 10]. But for the treatment of osteoporosis disease, it is necessary to be administered by intravenous injection that can lead to systemic functional improvement of bone quality. However, due to the rapid clearance of native protein drug *in vivo*, high dose and frequent administration usually have to be adopted to achieve therapeutic benefit. This can lead to high treatment cost and low patient compliance in chronic treatment. The short circulation time of NELL-1 *in vivo* could be one of the main limitations for the practical application of systemic therapy. Therefore, the main purpose of the present study was to extend the circulation time of NELL-1 *in vivo* by chemically modifying its molecular structure. Currently, one of the most popular technologies to prolong the half-life time of protein is to use water soluble polymers as a macromolecular carrier. As it is approved for human use by FDA, the non-toxic PEG molecule is widely used in numerous biomedical applications [11–13]. It is a water soluble polymer with excellent biocompatibility but without immunogenicity. PEG is commercially available in a wide range of molecular weights, which is particularly appropriate for the chemical attachment to proteins with various molecular weights. So it was chosen to conjugate with NELL-1 protein in the current study.

The methods of chemical modification of protein with PEG can be divided into two categories: site-specific conjugation and random conjugation. The site-specific conjugation method can produce better defined products using an N-terminal amine-specific or cysteine-specific PEGylation reaction. The N-terminal PEGylation often uses a PEGylating reagent with relatively low reactivity (such as PEG-aldehyde), since a high reactive PEG reagent will lead to an evident degree of lysine coupling [14]. Therefore, incomplete PEGylation and low yield were associated with this method. Cysteine-specific PEGylation can get a higher yield, but the problem is that the cysteine group of reduced form is rarely available in proteins because it is usually involved in disulfide bridges. Even naturally present, the cysteine group often plays an important role in protein structure or activity, and the

modification on it could lead to significantly reduced or lost bioactivity [15]. The approach of random conjugation is often used as the first method in many new PEG-protein studies since it is conventional and convenient. This could result in complex mixtures of various PEG-conjugate isomers differing both in the number of PEG molecules and the site of linking [16], but the advantage is that it is simple and can achieve sound PEG-conjugates with high yields. Furthermore, the PEG conjugate can be purified to produce a homogenous product.

To the best of our knowledge, no reports have been made on the PEGylation of NELL-1, a huge protein with the Mw much larger than all other proteins that have been PEGylated to date. In the present study, we PEGylated NELL-1 by random conjugation using three different PEG sizes (5, 20, 40 kDa). The PEGylated NELL-1 was synthesized using chemically activated PEG-N-hydroxysuccinimide (PEG-NHS) for conjugation with the amine group in lysine residue located at the surface of NELL-1. NHS was chosen for amine coupling reactions due to its high reactivity in bio-conjugation synthesis at physiological pH [17]. For each PEGylated NELL-1, the PEG modification degree, thermal stability, and cytotoxicity were determined. The *in vitro* bioactivity study of NELL-PEG was also evaluated in two primary cell lines, human perivascular stem cells (hPSC) and mouse calvarial osteoblast cells. Subsequently, the pharmacokinetic behavior of the PEGylated NELL-1 was examined in mice.

2. Material and Methods

2.1 Synthesis of PEGylated NELL-1

PEGylated NELL-1 (NELL-PEG) was synthesized from linear PEG-NHS Mw 5 kDa (PEG 5k, Sigma-Aldrich, USA), linear PEG-NHS Mw 20 kDa (PEG 20k, NOF America corporation, Japan), and 4-arm-branched PEG-NHS Mw 40 kDa (PEG 40k, NOF America corporation, Japan) according to a protocol described previously with modification [18]. Briefly, 0.5 mg of NELL-1 (10.0 mg/mL) was diluted to a concentration of 2.0 mg/mL in PBS buffer (pH6.5, 0.1 M), then added with 10 μ L of 62.5 mg/mL PEG 5k, or 40 μ L of 62.5 mg/mL PEG 20k, or 80 μ L of 62.5 mg/mL PEG 40k at a NELL-1 to PEG molar ratio of 1:100. The PEGylation system was reacted under 300 rpm magnetic stirring for 12 h at 4 °C. The obtained NELL-PEG was purified by loading the reaction mixture onto a Sephadex G-25M column (Sigma-Aldrich, USA), eluting the column with 3.0 mL of PBS solution (1x, pH 7.4), and collecting the fractions (0.25 mL/fraction) that mainly consisted of NELL-PEG determined by GPC method, then a 24 h dialysis against distilled water was performed using suitable dialysis cassettes (Fisher, USA) to remove any unreacted PEG molecules.

2.2 GPC characterization of NELL-PEG

The synthesis of PEGylated NELL-1 was confirmed by the gel permeation chromatography (GPC), and naked NELL-1 was used as control in the GPC analysis. Briefly, an ultra-hydrogel linear column (7.8 mm $\times$ 300 mm, 10 μ m grain diameter) was attached to a GPC system (Waters Corp.). The isocratic GPC investigations were performed using PBS buffer (50 mM, added with 0.15 M NaCl, pH 7.5) as mobile phase in a flow rate of 1.0 mL/min.

Ten μl of the protein solutions were injected and the GPC curve of the protein was recorded with a UV detector at 280 nm.

2.3 Fluorometric assay of PEG degree

The degree of PEG modification of NELL-PEG was determined by fluorometric assay with fluorescamine. Briefly, a fresh working solution of fluorescamine in DMSO at a concentration of 1.0 mg/mL was prepared first, and then a 12 μl of fluorescamine solution was added and mixed to 36 μl of NELL-PEG or naked NELL-1 in 0.1 M phosphate buffer (pH 8.0) in a 96-well clear bottom black micro-plate. A series of samples with different protein concentration was prepared and reacted for 15 min at 25 °C under gentle shaking. Then the fluorescence intensity was determined using a plate reader (Infinite F200, Tecan Group Ltd.) at an excitation wavelength of 390 nm and an emission wavelength of 475 nm. The naked NELL-1 was used as a control to determine the unreacted amine groups of the NELL-PEG.

2.4 Thermal shift assay

The thermal stability of NELL-PEG was evaluated by thermal shift assay method using a 7300 real-time PCR system (Applied Biosystems, CA). Prior to use, the environmentally sensitive fluorescent dye SYPRO Orange stock solution in DMSO (5,000x, Sigma) was diluted 1:125 in PBS. The samples were prepared in a 96-well plate in triplicate containing 3 μl of NELL-PEG (1.0 mg/mL), 2.5 μl of freshly diluted SYPRO orange (40x) and 19.5 μl of PBS buffer (0.01 M, pH 7.4), then the plate was sealed with optical quality sealing tape and centrifuged at 4,000 rpm for 2 min. The fluorescent intensity was monitored as the plate was heated from 298 to 368 K in an increment of 1 K/min. The fluorescent data was analyzed using a Boltzmann model and the melting point (T_m) was calculated.

2.5 Cytotoxicity assay of NELL-PEG

The cytotoxic effect of PEGylated NELL-1 and naked NELL-1 was tested by Alamar blue assay. MC3T3 cells were seeded in 96-well cell culture plates at a density of 10,000 cells/well and kept overnight in growth medium at 37 °C in a 5% CO₂ incubator. Then 100 μl of different concentration of NELL-PEG and naked NELL-1 medium were added into the cells and incubated for 24 h at 37 °C. MC3T3 cells without NELL-1 protein were used as negative control for 100% cell viability. Then the medium was replaced by growth medium containing 10% Alamar blue, and incubated for 1 h at 37 °C in dark. The fluorescent signal was measured by a plate reader (Infinite F200, Tecan Group Ltd.) with 560 nm excitation wavelength and 595 nm emission wavelength. The results were calculated by averaging the values obtained and subtracting the average value of no-cell control. The cytotoxicity of each NELL-PEG at each concentration was estimated in triplicate and analyzed statistically using one-way ANOVA for multiple comparisons.

2.6 Cells

2.6.1 Mouse calvarial osteoblast cells—The mouse calvarial osteoblast cells were isolated from calvaria (frontal and parietal bones) of 3–5 day old mice (strain C57BL/6). Briefly, the calvaria were harvested aseptically and the periosteal layers on both sides were

carefully stripped off with blade under sterile PBS solution, then rinsed and cut into trivial bone blocks using scissors. The bone blocks were immersed into 5 mL of trypsin-collagenase solution (DMEM containing 0.1% collagenase type I and 0.125% trypsin), incubated under a 150 rpm shaker at 37 °C for 15 min. Then the supernatant containing cells was transferred into a 50 mL centrifuge tube and spun down the cells at 1,500 rpm for 5 min. The supernatant was discarded and the cells were suspended in growth medium (DMEM containing 10% FBS, 50 U/mL penicillin and 50 ng/mL streptomycin). The cell extraction process was repeated 5 times. The obtained cells were pooled and cultured in growth medium at 37 °C incubator with 5% CO₂. After reaching 80% confluence, cells were split and passage 2 was used to conduct *in vitro* bioactivity experiment.

2.6.2 hPSC cells—The human perivascular stem cells (hPSC) were isolated from fresh adipose tissues as described before [19]. The cells were cultured in growth medium (DMEM containing 20% FBS, 50 U/mL penicillin and 50 ng/mL streptomycin) in a sterile incubator at 37 °C and 5% CO₂. Once reached 80% confluence, the cells were passaged and passage 2 was used to conduct *in vitro* bioactivity experiment.

2.7 Bioactivity in vitro

2.7.1 ALP testing—The bioactivity of the PEGylated NELL-1 was determined by measuring its ability to increase the expression of alkaline phosphatase (ALP) in the mouse calvarial osteoblast cells. The cells were cultured for 9 days in osteogenic medium at 37 °C in a 5% CO₂ humidified incubators, then the cells were solubilized in 200 µl lysis buffer (0.2% NP40 plus 1.0 mM magnesium chloride) for 15 min at 4 °C. The cell lysate was scrapped and centrifuged for 5 min at 12,000 rpm, then 15 µl of cell supernatant was mixed with 200 µl of ALP substrate buffer consisting of 0.4 mg p-nitrophenol phosphate, 100 µl alkaline buffer A (A9226, sigma) and 100 µl distilled water. After incubated for 15 min at 37 °C, 30 µl of 1.0 N NaOH was added to stop the reaction, then followed by colorimetric detection at 405 nm. Protein amount in the corresponding well was determined by Bradford assay. The quantitative analysis of ALP activity was normalized by the OD405 values of the ALP data to the corresponding protein amount. The measurements were performed in triplicate for each sample.

2.7.2 Mineralization testing—The mineral formation of calcium phosphate of the hPSC cells in osteogenic medium was assessed using Alizarin Red S (ARS) staining as described in a previous paper [20]. Briefly, the hPSC cells were incubated on a 24-well plate in osteogenic medium consisting of α -MEM, 10% FBS and mineralization-inducing components including L-ascorbic acid (50 mg/mL, Sigma, US) and β -glycerophosphate disodium salt (10 mM, MP Biomedicals, US). The medium was changed every 3 days. After 15-day incubation, the cell monolayers were washed with 2 mL PBS (1x, pH 7.4) per well, then fixed with 1 mL of 10% formaldehyde for 15 min. Next, the fixed cells were washed 3 times with distilled water and stained with ARS solution (40 mM, pH 4.2) for 20 min, then excess dye was removed by washing 4 times with distilled water, and the wells with stained mineral nodules were imaged by digital camera. Then the ARS in cells was leached by heating to 85 °C for 10 min in 10% acetic acid. The cell lysate was centrifuged at 14,000 rpm for 10 min, then the supernatant was collected and adjusted to pH 4.1–4.5 with 10%

ammonium hydroxide. The amount of ARS in each well was quantified at 450 nm. The experimental conditions were conducted in quadruplicate.

2.8 In vivo study

2.8.1 Preparation of FITC- labeled NELL-1 and NELL-PEG—The NELL-1 and PEGylated NELL-1 were labeled with fluorescein isothiocyanate (FITC) for *in vivo* study. Briefly, 50 µg of FITC (Sigma) was added into 0.25 mL of NELL-1 (4.0 mg/mL) in a 0.1 M sodium carbonate-bicarbonate buffer (pH 9.0) at a 50:1 molar ratio of FITC to protein, and reacted for 3 h under magnetic stirring at 250 rpm at room temperature. The FITC tagged NELL-1 was separated from unreacted FITC by passing through a Sephadex G-25 column, and the fractions containing FITC-NELL-1 were collected and pooled. The concentration and the degree of labeling (fluorescein/protein ratio, F/P) of the conjugate was determined by measuring its absorbance at 280 nm and 495 nm using a spectrophotometer. The vial of labeled protein was wrapped with aluminum foil to protect from light and stored at -20°C . The FITC labeled NELL-PEGs were prepared with the same procedure as above.

2.8.2 Animal and pharmacokinetic study—The 3-month-old female mice (CD-1 strain, Charles River Laboratories) were used to determine the residence of PEGylated NELL-1 *in vivo*. They were housed under laboratory conditions, and the experiment protocols for animal studies were approved by the UCLA Chancellor's Animal Research Committee.

The mice were randomly divided into 4 groups, 6 mice for each group. Group 1,2,3,4 was injected with FITC-NELL-1, FITC-NELL-PEG-5k, FITC-NELL-PEG-20k and FITC-NELL-PEG-40k, respectively. Each mouse was administered with 100 µl single IV bolus dose of protein (1.25 mg protein/kg of mouse body weight) from the lateral tail vein. The blood samples were drawn from the mouse retro-orbital sinus with a capillary tube at 0.5 and 24 h, then transferred into serum separator tubes. The serum was separated by centrifugation at 10,000 rpm for 5 min. The concentration of NELL-1 or NELL-PEG in serum was analyzed by monitoring the fluorescence intensity of FITC using a plate reader (Infinite F200, Tecan Group Ltd.). The blood tubes were wrapped with aluminum foil to protect from light during sample process.

2.9 Statistical analyses

Statistical analyses were performed using one-way ANOVA for multiple comparisons and Student's t-test for two-group comparisons at 95% confidence levels. Data are expressed as means $\pm$ SD, and value of (*) $P < 0.05$ was considered statistically significant differences, (**) $P < 0.001$ was considered statistically highly significant differences.

3. Results

3.1 Synthesis of PEGylated NELL-1

Three sizes of PEG were used in the experiment: the linear mPEG-NHS Mw 5 kDa, linear mPEG-NHS Mw 20 kDa, and 4-arm-branched mPEG-NHS Mw 40 kDa. During conjugation, the N-hydroxysuccinimide group of PEG-NHS was covalently reacted to the ε -

amine group presented on lysine side chains or the α -amine group of the NELL-1 protein. The reaction mechanism was illustrated in Fig. 1a. The schematic structures of the three PEGylated NELL-1 with different PEG molecules were shown in Fig. 1b. The PEGylation reaction was successfully confirmed by GPC characterization (Fig. 1c). The GPC system with a size exclusion column can separate different proteins on a basis of size. The NELL-PEG with larger size came out earlier and possessed a shorter retention time (RT). Fig. 1c showed the elution curves of the three NELL-PEG conjugates. The RT of NELL-1, NELL-PEG-5k, NELL-PEG-20k and NELL-PEG-40k were 8.614, 8.221, 7.632 and 7.876 min, respectively. The RT of PEGylated NELL-1 was smaller than that of the naked NELL-1, which indicated that the PEG molecules were successfully linked to NELL-1 and led to an increased size. The symmetrical peak shape of the NELL-PEG and no PEG peak observed in GPC profiles suggested the purification process was effective in the production. Therefore, the GPC characterization indicates that the chemical synthesis of NELL-PEG was successful.

3.2 PEG modification degree of PEGylated NELL-1

The PEGylation degree of the NELL-PEG was determined by the fluorescamine method as described above. The plots were shown in Fig. 2a. The regression coefficients for all the groups were close to 1. The amount of the residual amine group and PEG modification degree of each NELL-PEG were calculated based on the slopes of the linear regression analysis, and the results were listed in Fig. 2b. It can be seen that the PEG degrees of the NELL-PEG-5k, NELL-PEG-20k, and NELL-PEG-40k were 72.6%, 46.9% and 14.2%, respectively. The results showed that the PEG degree of NELL-PEG decreased as the molecular weight of PEG increased. This is because the NHS group in linear PEG with smaller Mw will more favorably react with the amine group in NELL-1 than larger Mw PEG, especially for the PEG with branched structure. The average molecular weights of the PEGylated proteins were estimated by the data of PEG modification degree. The results indicated that the NELL-PEG-20k monomer possessed the highest molecular weight among the three conjugates. The Mw of NELL-PEG-20k was about 4.1 times of naked NELL-1, 1.9 times of NELL-PEG-5k, and 1.4 times of NELL-PEG-40k.

3.3 Thermal stability of PEGylated NELL-1

The thermal stability of the PEGylated NELL-1 was investigated by the thermal shift assay. The fluorescent curves of different NELL-PEG and naked NELL-1 were shown in Fig. 3a. The T_m of the proteins analyzed by a Boltzmann model and the corresponding thermal shift amount ($T_m = T_m - T_0$) was listed in Fig. 3b. The T_m of naked NELL-1 was 49.75 °C. After PEGylation, the T_m was increased to 63.42, 50.51, 53.57 °C for NELL-PEG-5k, NELL-PEG-20k, and NELL-PEG-40k, respectively. The protein melting point of the NELL-1 was shifted to higher temperature after PEGylation, which suggested the stability of the NELL-1 protein was enhanced. Interestingly, the thermal stability of NELL-PEG-5k was much higher than the NELL-PEG-20k and NELL-PEG-40k. This can be explained by the difference in PEG modification degrees. A NELL-1 molecule linked with more PEG molecules can lead to higher stability. The PEG degree assay confirmed that each NELL-PEG-5k molecule possessed 31.5 PEG molecules, which was much higher than the 20.4 PEG molecules of NELL-PEG-20k and 6.2 PEG molecules of NELL-PEG-40k.

3.4 Cytotoxicity of PEGylated NELL-1

In this study, the effect of PEG type and concentration on NELL-PEG cytotoxicity was investigated in MC3T3 cell line by Alamar blue assay, and the result was shown in Fig. 4. Compared to the control group, the PEGylated NELL-1 and naked NELL-1 tested at concentrations up to 50 µg/mL did not show appreciable cytotoxicity to MC3T3 cells. After analysis using one-way ANOVA by SPSS, there was no significant difference between different NELL-PEG groups. The results indicate that the treatment of all the PEGylated NELL-1 at all concentrations is nontoxic to MC3T3 cells. In addition, no cytotoxic effect of NELL-PEG on primary hPSCs and mouse calvarial cells was observed in the following osteoblastic differentiation experiments.

3.5 Pro-osteoblastic bioactivity of PEGylated NELL-1 in vitro

3.5.1 ALP of mouse osteoblast cells—To examine the bioactivity of the NELL-PEG conjugates, their ability to facilitate the differentiation of the mouse osteoblast cells *in vitro* was investigated by ALP testing. The mouse osteoblast cells were incubated for a 9-day exposure to NELL-1 or NELL-PEGs in osteogenic medium, then the ALP activity of the cells was normalized on the basis of protein content in each well. The relative bioactivity of the NELL-PEG was shown in Fig. 5a. The result showed that three kinds of NELL-PEG did not show significant difference after analysis by one-way ANOVA with multiple comparisons, but their bioactivity was significantly higher than the control group without NELL-1 (* $p < 0.05$). Therefore, the PEGylated NELL-1 significantly enhanced the ALP activity of mouse osteoblast cells compared to control group.

3.5.2 Mineralization of hPSC—Subsequently, the ability of the NELL-PEG to facilitate the hPSC mineralization *in vitro* was investigated. After 15-day incubation in osteogenic medium, the hPSC cells were stained with ARS solution. Fig. 5d depicted the images of the whole wells containing stained calcium mineralization, which showed that all the protein groups (naked NELL-1, NELL-PEG-5k, NELL-PEG-20k, and NELL-PEG-40k) significantly increased the matrix mineralization compared to the negative control group (NELL-1 free). The ARS in the wells was next extracted from the hPSC cell monolayer by acetic acid for bioactivity quantification (Fig. 5b). The result showed that the bioactivity of the NELL-PEG measured by mineralization testing with hPSC cells was similar to the ALP testing with mouse osteoblast cells. The bioactivity of the three kinds of NELL-PEG was significantly higher than the NELL-1 free control group (* $p < 0.05$). The relative activity of different PEGylated NELL-1 protein compared to naked NELL-1 was listed in Fig. 5c. The NELL-PEG exhibited slightly decreased bioactivity compared to naked NELL-1, although they did not show significant difference after statistical analysis by SPSS. The decreased bioactivity of the NELL-PEG groups (especially for the NELL-PEG-40k) suggested that the PEGylation affected the bioactivity of NELL-1. Because more protein surface can be covered by branched PEG, the 4-arm branched PEG-40k could provide better protection for NELL-1 against attack by enzymes and other proteins *in vivo* [21], but it also hindered the interaction between cells and NELL-1 molecules, thus leading to the reduced bioactivity. The experiment shows that the bioactivity of the PEGylated NELL-1 was preserved and still can facilitate the osteogenesis of hPSC cells.

3.6 Circulation time of PEGylated NELL-1 in mice

The PEGylated NELL-1 and naked NELL-1 were labeled with FITC and used for monitoring their concentrations in blood. The F/P ratio of the obtained FITC-protein was 4.36 after analysis. In order to check whether the PEGylation of NELL-1 can increase its circulation time, the pharmacokinetics of the naked NELL-1 and PEGylated NELL-1 in CD-1 mice were investigated. Fig. 6 showed the remaining amount (%) of NELL-1 and NELL-PEG in mice at different time points after a single intravenous injection. For the naked NELL-1, only $9.3 \pm 3.7\%$ of the initial dose was detected in mouse blood at 0.5 h, but for the PEGylated NELL-1, the protein amounts at 0.5 h were significantly higher (NELL-PEG-5k, NELL-PEG-20k, NELL-PEG-40k were $22.6 \pm 8.3\%$, $67.1 \pm 3.9\%$, $44.0 \pm 4.3\%$, respectively). After 24 h administration, the amounts of PEGylated NELL-1 *in vivo* remained significantly higher compared to naked NELL-1, which was almost gone from the mice at that time. Furthermore, among the three kinds of NELL-PEG examined, the NELL-PEG-20k with linear 20 kDa PEG had significantly higher remaining amount in blood compared to the linear 5 kDa PEG and the branched 40 kDa PEG both at 0.5 h and 24 h. Therefore, NELL-1 PEGylation can significantly improve circulation time of NELL-1 *in vivo*.

4. Discussion

Osteoporosis is a progressive bone disease with the characteristic of a decrease in bone mass and density. There is greater osteoclast activity than osteoblast activity in patients, thus the net rate of bone resorption exceeds the rate of bone formation [22]. NELL-1 can specifically increase osteoblast differentiation and activity, but without a concomitant osteoclast response [7], which make it possible to be a promising therapy for osteoporosis treatment. Furthermore, unlike BMP-2, NELL-1 is a downstream mediator of runt-related transcription factor 2 (Runx2) during bone formation, thus less side effects occurred during application *in vivo* [1]. As a potent growth factor, NELL-1 has been studied preclinically for the induction of bone formation [1]. Because of the undesirable side effects of BMP-2 including excessive and ectopic bone formation, bone resorption, etc. [23, 24], NELL-1 protein has the potential of replacing BMP-2 in clinical application. Similar to other native protein drugs, NELL-1 has a relatively short half-life time *in vivo* after intravenous injection due to rapid clearance from the body, which limits its application as a therapy for osteoporosis. Meanwhile, the systemic nature of osteoporosis has called for intravenous medication administration as a therapeutic remedy. Therefore, in the present study, the circulation time of NELL-1 was extended by PEGylation in order to meet the demand of the osteoporosis treatment. PEGylation technology can improve the elimination half-life time of native proteins by preventing their renal clearance and decreasing protease degradation *in vivo* [25].

In this study, the effect of PEGylation on the circulation time of NELL-1 in mice was examined in order to determine whether it can improve the drug delivery *in vivo* or not. We first synthesized NELL-1 with three different PEG sizes (5, 20, and 40 kDa), and investigated their physical properties with GPC, fluorometric assay and thermal shift assay, then evaluated their cytotoxicity and bioactivity *in vitro* and pharmacokinetics *in vivo*.

GPC analysis of the PEGylated NELL-1 was conducted in order to monitor the synthesis and characterize the obtained conjugates. The decreased retention time of the PEGylated NELL-1 compared to naked NELL-1 indicated that they were successfully obtained by the adopted PEGylation method. Interestingly, the retention time of NELL-PEG-40k with branched PEG molecule was smaller than that of NELL-PEG-20k with linear PEG, indicating that the size of the NELL-PEG-40k was hydrodynamically smaller than the NELL-PEG-20k. The fluorometric assay data shown in Fig. 2b confirmed that the molecular weight of the NELL-PEG-40k monomer was smaller than NELL-PEG-20k. In addition, It is reported that the conjugated PEG moiety in PEGylated protein is still highly extended, similar to the random-coiled unconjugated PEG [26, 27], therefore, the NELL-PEG is likely to assume a highly elongated conformation, as depicted in Fig. 1b. Each chain of the 4 arm-branched PEG-40k actually is 10 kDa, which is shorter than the linear PEG-20k when they are in the highly extended conformation. This also explains why the hydrodynamic size of NELL-PEG-40k is smaller than NELL-PEG-20k.

The selected PEGylation method in current research is a random reaction, by which a PEG molecule can be coupled to all the accessible secondary NH_2 groups of lysine located at the surface of NELL-1 protein, which implies that one or multiple PEG molecules can be linked to each NELL-1 molecule. Therefore, it is necessary to quantify the number of PEG molecules coupled to NELL-1. The result of the fluorometric assay showed that there were 43.4 lysine residues accessible for chemical modification in each NELL-1 monomer. After PEGylation with different PEG molecules, the PEG modification degrees of NELL-PEG-5k, NELL-PEG-20k and NELL-PEG-40k were 72.6%, 46.9% and 14.2%, respectively. Thus there are 31.5, 20.4 and 6.2 PEG molecules conjugated to each NELL-1 molecule on average for NELL-PEG-5k, NELL-PEG-20k, and NELL-PEG-40k, respectively.

The intrinsic thermodynamic stability of the naked NELL-1 and PEGylated NELL-1 was determined by the thermal shift assay. Basically, PEGylated NELL-1 has a higher T_m than the naked NELL-1 as shown in the Fig. 3. Therefore, covalently linked PEG moiety can interact and stabilize the conformation of the NELL-1 in the immediate microenvironment. Interestingly, the NELL-PEG-20k, with the highest Mw, did not possess the highest thermal stability, which indicates that the thermal stability is independent of the molecular weight of the PEG moiety. Plesner et al. [28] have investigated the effect of PEG size on the stability of PEG-BSA, and concluded that the larger size of PEG chain did not contribute more to the stability of protein, which is supported by our experiment. Although it is reported that the PEGylation effect on the thermal stability of protein depends on several factors including the PEG degree, the site of the polymer conjugation, and the specific local interaction between the PEG and protein surface [29, 30], it is difficult to predict the thermal stability of PEGylated protein, because PEGylation may increase the stability for some proteins [31–33], but it also could decrease the stability for other proteins [28, 34].

The PEG related toxicity is rare for PEGylated proteins based on previous studies [35–37]. In the current research, the PEGylated NELL-1 with different PEG structures did not show any toxicity on MC3T3 cells at various concentrations, which indicate that the NELL-PEG are safe for the biological experiment.

Subsequently, the bioactivity of NELL-PEG was assessed by a cell-based assay. PEGylation modification usually led to reduced protein bioactivity because of the PEG resistance between protein and targeted receptors [25]. In the current study, two kinds of primary cells, the hPSC cells and mouse osteoblast cells, were used to determine the pro-osteoblastic bioactivity of naked and PEGylated NELL-1. Similar results were obtained by the mineralization testing of hPSC cells and the ALP activity testing of mouse osteoblast cells. All the PEGylated NELL-1 can significantly facilitate the differentiation of hPSC and mouse osteoblast cells compared to control group (NELL-1 free). Although PEGylated NELL-1 showed a reduced biological activity (especially for the NELL-PEG-40k) compared to naked NELL-1, the data did not show a statistically significant difference after analysis. Therefore, the bioactivity of NELL-1 was preserved after PEGylation.

PEGylation was used to increase NELL-1's residence in circulation system in the current research, so the assessment of the pharmacokinetics of NELL-1 after PEGylation is vital for its efficiency over naked NELL-1. In the current study, the remaining amounts of NELL-PEG and NELL-1 in mice after a single intravenous bolus injection were examined. The study demonstrated that NELL-PEG had a significantly higher remaining amount compared to naked NELL-1 *in vivo* at 0.5 h and 24 h. The result indicated that PEGylated NELL-1 could reside in mice for a much longer time than naked NELL-1 under equal initial dose. There was almost no naked NELL-1 that could be detected in blood at 24 h, while the PEGylated NELL-1 still could be tracked at that time, especially for the NELL-PEG-20k, for which the remaining amount was still up to 24%. These data confirm that PEGylated NELL-1 can lead to a much longer circulation time *in vivo*. Of the various sized PEGylated NELL-1 molecules, the remaining amount and circulation time of NELL-PEG-40k were smaller than the NELL-PEG-20k in blood. The reason is that both Mw and hydrodynamic size of NELL-PEG-40k are smaller than the NELL-PEG-20k as confirmed by the fluorometric assay and GPC characterization.

5. Conclusion

In the present study, we have prepared three kinds of NELL-PEG conjugates that varied in the size and structure of PEG molecules: linear 5 kDa, linear 20 kDa and branched 40 kDa, and characterized their properties by *in vitro* and *in vivo* testings. Compared to naked NELL-1, all three NELL-PEG conjugates showed not only a preserved bioactive potency *in vitro*, but also a significantly improved circulation time *in vivo* observed in mice. Further studies are needed to evaluate the effect of PEGylated NELL-1 on the treatment of osteoporosis with animal models, and the long-circulating NELL-PEG conjugates with a maintained osteogenic activity represent a solid step toward to developing an effective osteoporosis therapy.

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Fig. 1.
a. Schematic graph of the synthesis of NELL-PEG by conjugating the NELL-1 to N-hydroxysuccinimide derivative of PEG (PEG-NHS) with different molecular weights (5, 20, 40 KDa) at mild condition. **b.** Schematic illustration of the structure of obtained PEGylated NELL-1 with different sizes of PEG moieties (linear PEG 5 kDa, linear PEG 20 kDa and branched PEG 40 kDa). **c.** GPC traces of the synthesized PEGylated NELL-1 and naked NELL-1 in PBS (50 mM PBS, 0.15 M NaCl, pH 7.5) using an UV detector. The smaller retention time of PEGylated NELL-1 compared to naked NELL-1 suggests the success of NELL-PEG synthesis.

Fig. 2.

PEG modification degree of different PEGylated NELL-1 measured by fluorometric assay.

a. Fluorescamine regression curves of PEGylated NELL-1 and naked NELL-1 after 15 min reaction in 48 μ l of 0.1 M PBS (pH 8.0) at 25 $^{\circ}$ C. **b.** Comparison of statistical parameters of the regression curves for different PEGylated NELL-1 and naked NELL-1. The PEG modification degree and the Mw of NELL-PEG monomer were calculated based on the regression curves. For the regression equations, Y stands for fluorescence intensity, X stands for protein concentration.

Fig. 3.

Thermal shift assay results of different PEGylated NELL-1 measured by thermofluor method. **a.** Thermofluor-based protein unfolding curve of PEGylated NELL-1 and NELL-1 in PBS buffer (0.01 M, pH 7.4), and the solid lines represented the nonlinear fits using a Boltzmann model. **b.** Comparison of the melting point (T_m) and thermal shift amount (ΔT_m) of different PEGylated NELL-1 or NELL-1. The T_m of NELL-PEG was higher than that of the native NELL-1, which suggested that the stability of the NELL-1 protein was enhanced after PEGylation. Results are shown as mean $\pm$ SD ($n=3$)

Fig. 4. Cytotoxicity of PEGylated NELL-1 and naked NELL-1 on MC3T3 cells as determined by the Alamar blue assay. The Cells were incubated with or without PEGylated NELL-1/naked NELL-1 at various concentrations for 24 h at 37 °C. The Cell viability (%) was calculated relatively to the positive control group (non-treated cells). The results suggest that the PEGylated NELL-1 is noncytotoxic to MC3T3 cells. Data are shown as mean $\pm$ SD ($n = 3$).

Fig. 5.

Effect of PEGylation on the pro-osteoblastic bioactivity of NELL-1 *in vitro*. **a.** NELL-1 bioactivity measured by ALP testing of mouse osteoblast cells. The cells were incubated with or without NELL-1, NELL-PEG at a concentration of 300 ng/mL for 9 days, and the ALP activity of the cells was normalized on the basis of protein content for each well. The result shows that PEGylated NELL-1 still can enhance the ALP activity of mouse osteoblast cells compared to control group (* $p < 0.05$). **b.** NELL-1 bioactivity measured by hPSC cell mineralization testing after 15-day incubation treated with or without NELL-1, NELL-PEG at a concentration of 300 ng/mL. **c.** The normalized bioactivity of different NELL-PEG compared to naked NELL-1. **d.** Images of the whole wells of mineralized hPSC cells stained by alizarin red. It can be seen that the PEGylation retains the pro-osteoblastic bioactivity of NELL-1. Data are shown as mean $\pm$ SD ($n = 3$).

Fig. 6.

Remaining amount (%) of PEGylated NELL-1 and naked NELL-1 following an intravenous injection in mice. A dose of 1.25 mg/kg protein was injected into mice by the tail vein, and blood samples were collected at 0.5 and 24 h. The concentration of PEGylated NELL-1 was analyzed using a spectrofluorometer. The study indicates that all the PEGylated NELL-1 have a longer circulation time compared to naked NELL-1 (* $p < 0.05$, ** $p < 0.001$). Data are represented as the mean $\pm$ SD ($n = 6$)