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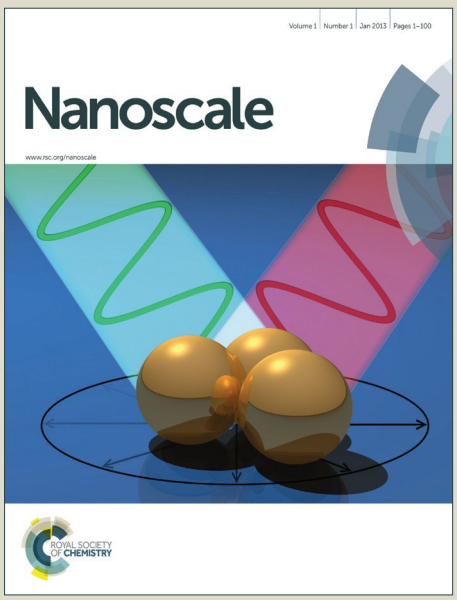

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Nanostructure controlled sustain delivery of human growth hormone using injectable, biodegradable, pH / temperature responsive nanobiohybrid hydrogel

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Abstract: The clinical efficacy of a therapeutic protein, human growth hormone (hGH), is limited by its short plasma half-life and premature degradation. To overcome this limitation, we proposed a new protein delivery system by self-assembly and intercalation of negatively charged

hGH onto a positively charged 2D-layered double hydroxide nanoparticle (LDH). The LDH-hGH ionic complex, with an average particle size of approximately 100nm, retards hGH diffusion. Nanobiohybrid hydrogels (PAEU/LDH-hGH) were prepared by dispersing LDH-hGH complex into a cationic pH- and temperature-sensitive injectable PAEU copolymer hydrogel to enhance sustained hGH release by dual ionic interactions. Biodegradable copolymer hydrogels comprising poly(β-amino ester urethane) and triblock poly(ϵ-caprolactone-lactide)-poly(ethylene glycol)-poly-(ϵ-caprolactone-lactide) (PCLA-PEG-PCLA) were synthesized and characterized. hGH was self-assembled and intercalated onto layered LDH nanoparticles through an anion exchange technique. X-ray diffraction and zeta potential results showed that the LDH-hGH complex was prepared successfully and that of the PAEU/LDH-hGH nanobiohybrid hydrogel had a disordered intercalated nanostructure. The biocompatibility of the nanobiohybrid hydrogel was confirmed by an in vitro cytotoxicity test. In vivo degradation of pure PAEU and its nanobiohybrid hydrogels were investigated and showed a controlled degradation of PAEU/LDH nanobiohybrids compared with pristine PAEU copolymer hydrogel. The LDH-hGH loaded injectable hydrogels suppressed the initial burst release of hGH and extended the release period for 13 days *in vitro* and 5 days *in vivo*. The developed nanohybrid hydrogel has the potential for application as a protein carrier to improve patient compliance.

Keyword: Human growth hormone, layered double hydroxide, injectable hydrogel, protein delivery.

Introduction:

Protein therapeutics have gained remarkable use in treating cancer, diabetes, inflammatory diseases, and abnormal growth because of their high target-specificity, reduced potential to

interfere with normal biological processes, and fewer side effects compared with small-molecule drugs[1] Sustained and prolonged release of therapeutic proteins, such as human growth hormone (hGH), in the therapeutic range *in vivo* is an important concern. Human growth hormone, a 191-amino-acid polypeptide, stimulates growth and differentiation of target tissue[8] and has been used clinically to treat child growth disorders, adult growth hormone deficiency, and growth failure caused by Turner syndrome or chronic renal failure[9,10]. Therapeutic proteins are highly disease-specific with no side effects. However, their intrinsic properties, such as a short biological half-life and poor absorbability in the gastrointestinal tract *in vivo*, lead to a need for daily subcutaneous injections, which may cause renal toxicity and poor patient compliance[11-13]. To surmount these issues, much attention has been paid to developing protein delivery systems that could maintain plasma protein concentrations within the therapeutic window over an extended period of time. In addition, these delivery systems could protect the therapeutic protein from premature degradation. These measures could enhance protein therapeutic efficacy and avoid frequent administration.

To develop these delivery systems, extensive efforts have been made to control hGH delivery mainly by crystal formulation, PEGylation, and loading it into microspheres or hydrogels[14-19]. PLGA microparticles are the most investigated method of long-term hGH delivery[17,20]. However, this system has disadvantages including an initial bust release, low protein loading, and use of organic solvent that may denature the protein[21]. A commercially available hyaluronate microparticle carrier provides sustained hGH release, maintaining serum hGH levels for 30 h in cynomolgus monkeys[9]. Injectable, temperature-sensitive hydrogels that exist as a sol at certain temperatures[22-25], which facilitates formulation with therapeutic agents[26, 27], but change to a gel at physiological temperature (37^{o}C) have also been investigated for hGH protein delivery[28-31]. hGH

is released from hydrogels in a short time due to its hydrophilicity and non-ionic interactions with hydrogel systems. To improve these systems, several groups have tried to incorporate a polyelectrolyte complex of protamine with hGH in a hydrogel matrix to minimize the initial burst release and allow sustained hGH release for up to 3 days invivo[32]. Recently, pH- and temperature-sensitive hydrogels have become promising carriers to deliver proteins/drugs because the hydrogels form ionic interactions with therapeutic agents and have many advantages for delivering ionic drugs/proteins[33-42]. Copolymer solutions exist in the sol state at low pH (cationic hydrogels) or high pH (anionic hydrogels) but turn into gels under physiological conditions (37^{o}C, pH 7.4). Our group proposed using cationic, pH- and temperature-sensitive hydrogels as *in vivo* release carriers for hGH. While hGH release within the therapeutic window has been demonstrated for up to 3 days,[43, 44] new, prolonged, sustained delivery formulations of hGH are needed to improve patient convenience.

The development of new pH- and temperature- sensitive nanobiohybrid injectable hydrogels represents a viable strategy to mimic the complex, hierarchical structure of native tissue and provide prolonged, sustained protein release and enhance protein therapeutic efficacy, which could revolutionize health care. The main aim of this work was to develop an injectable biodegradable nanobiohybrid hydrogel for sustained hGH release over a long time to improve patient convenience. A new protein delivery system was proposed by intercalating and absorbing hGH inside the layered galleries of positively charged, 2D-layered, double hydroxide nanoparticles (LDH). A nanobiohybrid hydrogel (PAEU/LDH-hGH) was prepared by dispersing LDH-hGH complex into acationic pH- and temperature- sensitive injectable PAEU hydrogel to enhance sustained hGH release by dual ionic interactions with LDH nanoparticles and cationic copolymer. Biodegradable pH- and temperature-sensitive copolymer hydrogels comprising

poly(β-amino ester urethane) and triblock poly(ϵ-caprolactone-lactide)-poly(ethylene glycol)-poly-(ϵ-caprolactone-lactide) (PCLA-PEG-PCLA) were synthesized and characterized. hGH was intercalated into layered LDH nanoparticles by anion exchange. X-ray diffraction patterns and zeta potential results showed that the LDH-hGH complex was prepared successfully and that the PAEU/LDH-hGH nanobiohybrid hydrogel had a disordered intercalated nanostructure. The *in vitro* cytotoxicity of the nanobiohybrid hydrogel was evaluated, confirming excellent biocompatibility of the nanobiohybrid hydrogel. *In vivo* controlled degradation of pristine PAEU hydrogel were measured along with LDH nanoparticles. The LDH-hGH-loaded injectable hydrogels suppressed the initial burst release of hGH and had an extended release period of 13 days *in vitro* and 5 days *in vivo*. Overall, our results suggest that injectable, biodegradable, and pH- and temperature-sensitive PAEU nanobiohybrid hydrogel has great potential as an effective delivery mechanism for sustained hGH release to improve patient compliance.

Experimental:

Materials:

Poly(ethylene glycol)s (PEGs), ϵ-caprolactone (CL), D,L-lactide (LA), 1,6-hexamethylene diisocyanate (HDI), Tin-2-methyloctoate ($Sn(Oct)_2$), dibutyltin dilaurate (DBTL), anhydrous chloroform ($CHCl_3$), anhydrous dicloromethane (DCM), 1-(2-hydroxyethyl) piperazine (HP), and phosphate buffered saline (PBS) were purchased from Sigma-Aldrich. 4-hydroxybutyl acrylate (HBA) was obtained from TCI Co. (Tokyo, Japan) and used as received. Diethyl ether, n-hexane, sodium hydroxide (NaOH), and hydrochloric acid (HCl) were supplied by Samchun Co. (Seoul, Korea). All other chemicals were analytical grade and used without any further purification.

Synthesis of pH- and temperature-sensitive multiblock copolymer PAEU:

i. *Synthesis of triblock PCLA-PEG-PCLA:*

A thermosensitive triblock copolymer of PCLA-PEG-PCLA was synthesized by ring-opening copolymerization of LA and CL in the presence of PEG as an initiator and $Sn(Oct)_2$ as a catalyst (**Scheme 1a**). In a dry, 250mL two-neck flask, 10.25g of PEG (M_n = 2050) and 0.01g of $Sn(Oct)_2$ were dried under vacuum at 110°C for 2 h. After that the temperature was decreased to 60°C, and 5.91mL of CL and 3.87g of LA were added and dried under vacuum at 60°C for 1 h. Before the main reaction step, the flask was filled with dried nitrogen and temperature was slowly raised to 130°C. The reaction was carried out at 130°C for 24 h. After the reaction finished, the product was dissolved in chloroform and precipitated in a 50/50 volume mixture of diethyl ether/n-hexane. The precipitated product was dried under vacuum at 25°C for 48 h.

ii. *Synthesis of monomer amino ester dihydroxyl HPB:*

Monomer amino ester dihydroxyl HPB was synthesized as described previously[39]. Briefly, HP (20mmol) was dissolved in anhydrous DCM (40mL) at room temperature before reacting with HBA (20mmol) at 45°C for 3 h.The mixture was then concentrated by vacuum evaporation of DCM and precipitated in cold n-hexane (**Scheme 1b**). The filtered product was dried under vacuum at 25°C for 48 h before use.

iii. *Synthesis of multiblock copolymer $(PCLA\text{-}PEG\text{-}PCLA\text{-}PAEU)_x$, briefly named PAEU:*

Multiblock copolymer PAEU was synthesized by forming a urethane bond between isocyanate groups of HDI, hydroxy groups of PCLA-PEG-PCLA, and monomer HPB in the presence of DBTL as a catalyst (**Scheme 1c**). In a dry, two-neck, round-bottom flask, 9g of PCLA-PEG-PCLA (1 mol equiv.) and 0.032g of DBTL were added and dried under vacuum at 80°C for 2 h. Then, 4.66g of HPB (7 mol equiv.) was added and dried at 60°C for 30 min. After charging the

flask with nitrogen, the reactants were dissolved in anhydrous chloroform to make a 10% weight solution. Subsequently, 3.18mL HDI (8 mol equiv.) was added, and the reaction was carried out at 60°C under nitrogen for 3 h. Finally, the mixture was evaporated and precipitated in diethyl ether to obtain the final product. The precipitated copolymer was dried under vacuum at room temperature for 48 h.

Characterization of multiblock copolymer (PCLA-PEG-PCLA-PAEU):

A 500-MHz spectrometer (Varian Unity Inova 500NB instrument) was used to record ^{1}H-NMR spectra with $CDCl_3$ as the solvent to determine the chemical composition of the product. Futecs GPC system containing an isocratic pump (FutecsNS2001P), a column temperature controller (Futecs AT-4000), a refractive index detector (Shodex, RI-101) and a series of three Shodex GPC columns (K-804, K-803, K-802) were used to measure the molecular weight and polydispersity index (PDI) of the copolymer. $CHCl_3$ was the eluent for GPC at aflow rate of 1.0 mL min^{-1} at 35°C. Polyethylene glycol standard samples were used for calibration. The characteristics of synthesized PCLA-PEG-PCLA triblock and PAEU multiblock copolymer are listed in **Supplementary Table 1**.

Preparation of layered double hydroxide nanoparticles and PAEU nanobiohybrids:

Mg_2Al-LDH-Cl nanoparticles (LDH) were prepared as previously described[45]. Briefly, LDH nanoparticles were prepared by vigorously mixing 10mL of salt solution containing $MgCl_2$ (2.0 mmol) and $AlCl_3$ (1.0 mmol) with 40 mL NaOH (0.15 M) and stirring for 30 min at room temperature under nitrogen. After nanoparticles were collected by centrifugation, they were dispersed in deionized water (40 mL) and hydrothermally treated at 100°C for 16 h.

To achieve a good dispersion, intercalation, and absorption of human growth hormone onto LDH, nanoparticles were sonicated in deionized water containing human growth hormone. The nanohybrid hydrogels were prepared through a solution route by dissolving copolymer PAEU in PBS at low pH and increasing the pH of the solution to 6.0. The dispersed LDH nanoparticles and PAEU solution were mixed to prepare the nanobiohybrid hydrogel. The hydrogel pH was maintained with 5 N NaOH and 5 N HCl, and it was stabilized at 2°C overnight. Henceforth, we call the hGH and LDH complex LDH-hGH and the corresponding nanobiohybrid hydrogel PAEU/LDH-hGH. In both cases, 2wt% LDH and 15wt% copolymer were used to prepare the nanohybrid hydrogels.

XRD characterization:

X-ray diffraction experiments were performed using a Bruker HP D8 Advance wide-angle X-ray diffractometer with CuK α radiation and a graphite monochromator (wavelength, λ = 0.154nm). The generator was operated at 40kV and 100mA. LDH nanoparticle and nanobiohybrid hydrogels were placed on a quartz sample holder at room temperature and scanned diffraction angle 2θ from 1° to 40° at a scanning rate of $1°min^{-1}$.

Zeta potential measurement:

The ζ-potentials of the LDH nanoparticle, PAEU polymer, hGH, and nanobiohybrid PAEU/LDH-hGH complex were measured using a Meta sizer Nano ZS instrument (Malvern Instruments) at room temperature. The copolymer and nanobiohybrid complexes were prepared by mixing LDH, hGH, and copolymer solutionsat a ratio of 4:1:30 at pH 6.0.The pH of the complexes was adjusted to 7.4 with1 N NaOH and 1 N HCl and was stabilized at room temperature for 20 min before the measurements were taken.

Sol-gel transition phase diagram:

Sol (flow) state and gel (non-flow) state behaviors of the copolymer solution were determined with the vial inverting method. Generally, aqueous solutions of polymers were prepared at different pHs, and the sol-gel behavior was measured in a temperature-controlled water bath. The copolymer was dissolved in PBS at low pH (~2) and temperature at a given concentration. After the copolymer completely dissolved, the pH was altered to ~6 and a 2wt% LDH nanoparticle was mixed with copolymer solution to obtain copolymer nanobiohybrids. The pH of the copolymer solutions was adjusted with 5 N NaOH and 5 N HCl to the desired value and was stabilized at 2°C overnight. In a temperature-controllable water bath, all samples were placed and equilibrated at 2°C for 20 min before the temperature was slowly increased at 2°C intervals. The sol-gel behavior was observed by inverting sample vials and observing the flow and non-flow state after 1 min.

Rheological measurement:

The rheological properties of the copolymer solutions were investigated by measuring the change in complex viscosity with temperature. A dynamic mechanical analyzer (Bohlin Rotational Rheometer) was used in oscillation mode with controlled shear stress of 0.4Pa and frequency of 1 rad.s^{-1}. The copolymer solution was placed between a 20mm diameter upper plate and 100mm diameter bottom plate with a gap size of 250μm. Complex viscosity was measured at a temperature sweep from 5°C to 65°C with a heating rate of 2°C min^{-1}.

In vitro degradation:

The *in vitro* degradation of PAEU and its nanobiohybrid hydrogels were determined with the weight loss method. Briefly, 0.5mL of solution was prepared in a 4mL vial, and the pH was

adjusted to 7.4. The samples were stabilized overnight and incubated in a water bath at 37°C for 30min. After the gel was formed, 3mL PBS was added. PBS was exchanged daily and samples were collected at pre-determined intervals. Freeze-dried samples were weighed to measure degradation.

In vivo stability:

To determine biodegradation, *in vivo* stability was performed with male Sprague-Dawley (SD) rats. SD rats (5-6 weeks old, approximate weight 200g) were used in accordance with the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals (NIH publication 85-23 revised 1985). An aqueous solution of pristine PAEU copolymer and PAEU/LDH nanobiohybrid was prepared at 15wt%, and the pH was adjusted to 6.3 and stabilized overnight before use. Two hundred fifty micro liter of each solution was injected subcutaneously into the back of an SD rat. At pre-determined times, SD rats were sacrificed and gel photographs were collected with a digital camera. The *in vivo* gels were carefully removed with scissors and freeze-dried for 3 days. The *in vivo* stability and degradation rate were calculated from the remaining weight of the lyophilized samples.

In vitro cytotoxicity:

In vitro cytotoxicity of PAEU/ LDH nanohybrids against HCT-166 cells was assessed after exposing the cells to different concentrations (50−2000 μg/mL) of nanohybrids for 48 h. Fresh DMEM was used as a negative control. After the 48h incubation, cell viability and proliferation were determined with MTT assay. Briefly, 20 μl/ well of fresh growth medium of MTT was added to each well and the cells were incubated at 37°C for 2 h. After that, medium was removed from the well and replaced with DMSO for further incubation of 1h. The absorbance at 490nm

(SpectraMax1 M5 Microplate Reader, Molecular Devices, Inc.) was directly proportional to the number of living cells. The survival percentage relative to the mock-treated cells (100% survival) was calculated.

In vitro release of human growth hormone:

The controlled release of hGH from PAEU copolymer and the PAEU/LDH-hGH nanobiohybrid hydrogels was first examined in vitro. An aqueous solution of copolymer PAEU was prepared at a concentration of 15wt% and adjusted to pH 6.0. The copolymer solution was separated into two sets: one containing LDH-hGH and one without LDH solution. hGH solution was pre-mixed with the LDH-dispersed PBS solution and sonicated for 20 min to form an hGH intercalated LDH-hGH complex solution at the desired concentration. hGH solution and the hGH-LDH complex were mixed with polymer solution at 2°C for 3 h to fabricate polymer hGH (PAEU-hGH) and LDH-hGH polymer solution (PAEU/LDH-hGH), respectively. Then, the pH of all the samples was adjusted to 7.4 and stabilized overnight. Finally, the samples were incubated in a water bath at 37°C for 30 min with shaking at 30 rpm. Three milliliters of fresh PBS solution (at 37^{o}C, pH 7.4) was added to the sample vials, and contacted the gel surface. At a given time, 1.5mL of release medium was collected and exchanged with 1.5mL of fresh release medium. The hGH concentration in the release medium was evaluated with a BCA assays kit (Pierce, USA).

In vivo release of human growth hormone:

To demonstrate the potential of this nanobiohybrid system in controlled protein delivery, in vivo hGH release was measured in SD rats. Copolymer solution was administered in SD rats as described for thein vivo stability test. Twelve male SD rats were separated into 4 groups (4

rats/group): the first group (control group) was injected with hGH solution (5mg mL^{-1}),the second group was injected with hGH-loaded PAEU copolymer solution (PAEU-hGH, 5mg mL^{-1}), and the third group was injected with LDH-hGH-loaded PAEU copolymer solution (PAEU/LDH-hGH, 5mg mL^{-1}) and fourth group was injected with LDH-hGH complex, 5mg mL^{-1}.Three hundred microliter of sample at pH 6.3 was subcutaneously injected in the back of SD rats. At predetermined times, blood samples were drawn from the tail vein and centrifuged to obtain the sera, which were stored at -21^{o}C until assayed. A commercial immunoenzymetric hGH ELISA kit (hGH-EASIA, DIA source Immuno Assays S.A.,Belgium) was used to analyze the concentration of released hGH in the sera.

Result and discussion:

Synthesis and Characterization:

The final PAEU multiblock copolymer was synthesized by reacting 1,6-diisocyanate with hydroxyl groups on the triblock polymer PCLA-PEG-PCLA and monomer HPB in the presence of DBTL as catalyst. The structures of the synthesized triblock copolymer PCLA-PEG-PCLA, monomer HPB, and multiblock copolymer PAEU were characterized by using ^{1}H-NMR spectra. **Figures 1-a, 1-b,** and **1-c** show the ^{1}H-NMR spectra of the triblock copolymer PCLA-PEG-PCLA, monomer HPB, and multiblock copolymer PAEU, respectively. In **Figure 1-a**, the signals at 3.65 (a), 5.20 (b), and 2.30 (d) ppm were assigned to protons of PEG (CH_2-CH_2-O), LA (-CH(CH_3)-), and LA (-CO-CH_2-CH_2-), respectively. The composition of PCLA-PEG-PCLA was calculated by the relative ratios of these peak areas. The monomer HPB was synthesized by Michael addition reaction between HBA and HP. Peak b (2.66-2.74 ppm) in **Figure 1-b** confirms the formation of a new methylene group from this reaction. **Figure 1-c** shows the ^{1}H-NMR spectrum of the multiblock copolymer PAEU. Signals at 3.65, 5.20, and 2.30 ppm confirm the

presence of PEG, CL, and LA, respectively. The presence of HDI in the copolymer was demonstrated by the first methylene proton at signal 3.15 ppm (peak b). Peaks j, e, f, and k were assigned to the appearance of monomer HPB in the final copolymer. The number of AEU units in a PAEU molecular complex was calculated by the relative ratio of the areas of peak b and peak a. The GPC traces in **Figure 2** & **Supplementary table 1** present the molecular weight and polydispersity index (PDI) of PEG (MW = 2050), triblock PCLA-PEG-PCLA, and PAEU multiblock copolymer. Comparing the molecular weight of PEG, PCLA-PEG-PCLA, and PAEU indicated a shift in elution toward a lower retention time, indicating a higher molecular weight. GPC measurement showing one small peak for triblock PCLA-PEG-PCLA and PAEU multiblock copolymer demonstrated complete polymerization. These characterizations affirmed the successful synthesis of PAEU multiblock copolymer.

Physicochemical features of LDH, LDH-hGH complex, and the nanobiohybrid hydrogels:

Two-dimensional LDH nanoparticles with an appropriate ion exchange capacity and biocompatibility were prepared by co-precipitating mixed metal ions (Mg^{2+}/ Al^{3+} =2/1) with a base (0.1N NaOH, pH ~ 10) in aqueous solution under nitrogen. The resulting white precipitate was washed with decarbonated water several times, dried for structural analysis, and treated with hGH. With this method, we loaded hGH inside the 2D-layered double hydroxide (LDH) nanoparticles through the ion-exchange process. A well-dispersed, stable LDH-hGH suspension was obtained. Subsequently, the LDH-hGH complex was dispersed in a pH- and temperature-sensitive PAEU copolymer solution at pH 6.0, which changes to a gel at the higher pH and temperature of the body (pH 7.4, 37°C). The structural characteristics of LDH-hGH and PAEU/LDH-hGH nanobiohybrids were examined. As shown in **Figures3a & 3b** pristine LDH particles had a series of well-developed (00l) reflections, corresponding to (001), (003), and

(006) and demonstrating the formation of a crystalline layered structure. The basal spacing of LDH was 0.76nm, identical to the reported value [46, 47].The shift in the diffraction peak (001 plane) of LDH (d_{001}=1.5nm; 2θ =5.7^o) towards a lower angle (d_{001}=1.75 nm; 2θ =5.0^o) and markedly reduced diffractions at 2θ of 11.2^o (003) and 21.0^o (006) after association with hGH reveal that the stacked hydroxide layers were severely distorted, which could be attributed to extensive hGH intercalation into the LDH gallery, at least near the inter layer region. Subsequent to adding the LDH-hGH complex to the PAEU polymer hydrogel, the (001) peak disappeared, the (003) peaks shifted to a lower angle (d_{003} =1.62 nm, 2θ = 5.4^o), and the (006) peaks become broader due to sufficient interactions between the polymer and LDH-hGH complex. These changes indicate an exfoliated/disordered nanostructure. A schematic representation of LDH synthesis and hGH interaction in PAEU/LDH-hGH nanohybrid hydrogels is shown in **Figure 3c**. The predicted dual interaction in nanohybrid hydrogels arose from the positive charge of the tertiary amine group, and the negatively charged LDH-hGH complex provided a better interaction site, resulting in a disordered/delaminated nanostructure.

Particle size and zeta potential of pure LDH, PAEU, hGH, and the nanobiohybrids with different weight ratios are shown in **Figure 4a & b**. The average size of an LDH-hGH particle was 95-100 nm, which was larger than that of pristine LDH (50-80 nm). This result indicates that hGH successfully adsorbed through self-assembly onto the LDH surface. In addition, the zeta potential of pure LDH was positive (+40 mV) whereas the zeta potential became negative (-3 mV) after interaction with hGH, confirming that hGH extensively adsorbed on the surface. The larger lateral dimension of LDH-hGH compared with pure LDH, as evident from TEM micrographs **(Figure 4c**), confirmed that hGH successfully adsorbed and intercalated onto the LDH surface.

The hGH intercalated and absorbed onto LDH balanced the slightly negatively charge which can increase ionic interactions through cationic copolymer hydrogel PAEU.

Sol-gel phase transition diagram:

The sol-gel phase transition of the aqueous solution of PAEU copolymer and the PAEU/LDH-hGH nanobiohybrids was studied with the tube inverting method. As shown in **Figure. 5a,** the sol-gel transition of PAEU copolymer was driven by pH. The aqueous PAEU solution was in the sol (flow) state at low pH and the gel (non-flow) state at neutral and high pH with a transition boundary of pH 6.8. At low pH (such as pH 6.0), the tertiary amines in PAEU are ionized, making the copolymer more hydrophilic. Therefore, the aqueous solution remained in the sol state across the whole range of experimental temperatures. In contrast, the deionization of pH-sensitive tertiary amines at high pH enhanced micelle formation and interaction, creating a gel state (pH 7.4, 37^{o}C). The upper gel-to-sol transition temperature at neutral and high pH was due to the PEG chain dehydrating and hydrogen bonds between PAEU blocks breaking. Dispersing 2wt% LDH-hGH nanoparticles in the hydrogel matrix did not significantly alter the sol-gel transition boundary.

Rheological properties:

Mechanical properties of pristine PAEU and its nanobiohybrids in the sol and gel states were investigated by dynamic mechanical analyzer to confirm the sol-gel phase transition of the hydrogels. **Figure 5b** shows the change in complex viscosity of 15wt% solutions of PAEU copolymer and PAEU/LDH-hGH nanobiohybrids as a function of temperature at different pHs. At low pH (pH 6.3), both the copolymer and nanobiohybrid solutions had low viscosity (10 Pas) across the temperature range, confirming a sol state. At physiological pH (pH 7.4), high viscosity at low temperature indicated a gel state. The viscosity of PAEU/LDH-hGH nanobiohybrids

(4278 Pas at 37^{o}C) was dramatically higher than that of pristine copolymer (722 Pa s at 37^{o}C) in the gel state, indicating a better interaction between the nanoparticle and copolymer matrix. In other words, the gel strength of the copolymer hydrogel increased after nanohybrids formed. Furthermore, viscosity decreased at higher temperature (~55^{o}C) due to PEG dehydrating and the micelle network and hydrogen bonds breaking at high temperature.

In vitro cytotoxicity:

Injectable hydrogels are used for many biomaterial applications, including therapeutic compound delivery, 3D-scaffolds for tissue engineering, carriers for cell encapsulation, and adhesives or barriers between tissue and material surfaces[48, 49]. In particular, dispersing a nanometer-sized 2D-nanoparticle in an injectable hydrogel matrix to obtain nanohybrids provides a facile interaction to create surfaces with a wide range of chemical and physical properties. These nanohybrid hydrogels may induce biological responses or be potentially harmful for long-term implants. Therefore, different concentrations (50–2000 μg mL^{-1}) of the PAEU/LDH nanohybrid solution in PBS and fresh DMEM as a negative control were used to treat HCT-116 cells for 48 h, and the cytotoxicity was evaluated **(Figure6)**. PAEU/LDH nanohybrids were not cytotoxic, even at 2000 μg mL^{-1}. Cell viability was ~90% at nanohybrid concentration up to 2000 μg mL^{-1}. The results supported the conclusion that PAEU/LDH nanohybrid hydrogels do not exhibit any cellular toxicity, facilitating their use as a non-cytotoxic injectable material for *in vivo* applications.

In vivo gel formation and degradation:

In vivo gelation was measured in male SD rats to investigate gelation of the PAEU copolymer and nanobiohybrid hydrogels. The integrity of solid hydrogels was investigated within 15 min after subcutaneous injection into SD rats, and suggested that the hydrogel was injectable and biodegradable (**Supplementary Figure 1).** *In vivo* weight loss of pure PAEU and a PAEU/LDH

nanohybrid hydrogel was 20 wt% and 35wt%, respectively, after 35 days **(Figure 7a)**. This result shows that nanohybrids are slowly degraded after hydrogel formation. The shape of both hydrogels (PAEU & PAEU/LDH) remained stable for more than 4 weeks, and the size gradually decreased, possibly because of copolymer degradation, as observed in optical micrographs (**Figure 7b)**. The interior morphologies of pure PAEU and PAEU/LDH nanohybrid hydrogels before and after degradation were investigated by SEM (**Figure 7c)**. Before degradation, pure PAEU hydrogels had separate, big pores, and nanohybrid hydrogels had smaller fibrous pores. After degradation, the pore size of pristine PAEU hydrogels was more severely affected than that of PAEU/LDH nanohybrid hydrogels. Similarly, *in vitro* controlled degradation of nanohybrids hydrogel was also observed compare to pristine PAEU hydrogel (**Supplementary Figure 2)**. These results confirm that degradation of nanohybrid hydrogels is controlled. This controlled degradation might be due to higher mechanical strength and smaller pore size of the nanohybrid hydrogel. In conclusion, controlled/slow degradation was observed after incorporating LDH nanoparticle in a PAEU hydrogel, presumably because of the enhanced mechanical strength, as evident from the higher viscosity, and the smaller, interconnected pores in nanohybrid hydrogels.

In vitro hGH release:

The zeta potential of hGH loaded onto LDH at various weight ratios was characterized in PBS at pH 7.4 to determine suitable conditions **(Supplementary Figure3).** The zeta potential of pristine hGH solution was -14 mV. After mixing with LDH nanoparticles, zeta potential increased sharply with an increased LDH/hGH ratio. From the zeta potential measurement, we choose an LDH-hGH weight ratio of 4:1, which is negatively charged and can have a stronger ionic interaction with the cationic hydrogel. The zeta potential increased from -3.0 mV to ~0 mV after the LDH-hGH complex was dispersed into the cationic hydrogel at a PAEU/LDH-hGH ratio of

30:4:1. This result indicates the ionic interaction between LDH-hGH and the PAEU hydrogel that could be responsible for sustaining and prolonging hGH release **(Figure 4b**). To confirm the effect of ionic interactions between LDH-hGH nanoparticles and the cationic hydrogel on the hGH release kinetics, hGH release from pristine PAEU, LDH-hGH complex and LDH-hGH loaded nanoparticles in PAEU (i.e. PAEU/ LDH-hGH nanohybrid hydrogel) was measured **(Figure8**). hGH released from the PAEU/LDH-hGH nanohybrid hydrogel was sustained for 13 days compared with 8 days for pristine PAEU hydrogel. The sustained and prolonged hGH release from nanohybrid hydrogels arises from hGH intercalation and adsorption onto the LDH surface and the ionic interactions between hGH and LDH or the cationic copolymer. In addition, the small pore size, control degradation, and good mechanical strength of the nanohybridgel compared with pristine PAEU hydrogel also contributes to the prolonged release.

In vivo hGH release:

The final goal for this nanobiohybrids hydrogel is to deliver and control the hGH concentration in blood plasma in an effective therapeutic window over a prolonged time frame to solve the current limitation of hGH delivery. Nanohybrid hydrogel protein release systems have been tuned to prolong the release kinetics, regulate distribution, and minimize side effects, thereby enhancing the therapeutic efficacy of the protein. Sustained hGH delivery results from nanohybrid hydrogels is obvious compared with pristine PAEU hydrogels. Protein release from nanohybrid hydrogel is a factor of dual ionic interactions between the protein and the nanoparticle or copolymer and controlled biodegradation. Protein release from a matrix occurs in three distinct steps: liquid penetrates into the matrix, the protein dissolves, and the protein diffuses. Any of these processes can be rate determining. Therefore, we perform a pharmacokinetic study in SD rats using pure PAEU, LDH-hGH and PAEU/LDH-hGH. We

chose a threshold of 1ng mL^{-1}hGH. hGH is known to have biological efficacy in the range of 1-5 ng mL^{-1} in humans[50]. **Figure 9** shows the pharmacokinetic profiles of hGH alone (5mg mL^{-1}, control group), hGH-loaded pristine PAEU, LDH-hGH complex and PAEU/LDH-hGH hydrogel (hGH 5 mgmL^{-1}, 15wt% hydrogel) injected in the back of SD rats. With hGH alone, there was an initial burst release (C_{max} 500ng mL^{-1} at 1h) of human growth hormone and complete release within 12h. With LDH-hGH complex and pristine PAEU-hGH hydrogel, the serum hGH concentration remained high (~1 ngmL^{-1}) for 1 and 3 days respectively with a minimal initial burst release (C_{max} 55 & 27ng mL^{-1} for LDH-hGH and PAEU-hGH hydrogel) compared with hGH alone. This extended hGH release from the pristine hydrogel was mainly attributed to the formation of ionic complexes between the anionic hGH and cationic PAEU hydrogels. After dispersing the LDH-hGH complex into the hydrogel matrix (i.e. PAEU/LDH-hGH nanobiohybrid hydrogel), the initial burst release was further suppressed, and the duration of hGH release was dramatically increased to 5 days. These effects arose from hGH protection in the LDH gallery, dual ionic interactions with LDH and the hydrogel, and the smaller interconnected pores in the nanobiohybrid.

Conclusion:

We propose a new protein delivery system that uses an injectable, cationic, pH- and temperature-sensitive PAEU hydrogel loaded with hGH intercalated with a layered double hydroxide nanoparticle complex (LDH-hGH) to achieve both a controlled, sustained release and increased bioavailability of hGH. Biodegradable pH- and temperature-sensitive copolymer hydrogels composed of poly(β-amino ester urethane) and triblock poly(ϵ-caprolactone-lactide)-poly(ethylene glycol)-poly-(ϵ-caprolactone-lactide) (PCLA-PEG-PCLA) were synthesized and characterized. Self-assembly and hGH intercalation inside the layered LDH nanoparticles were

achieved by anion exchange technique. Zeta potentials showed that the LDH-hGH complex formed successfully because of its ionic interaction. Nanobiohybrid PAEU/LDH-hGH hydrogels were prepared by dispersing the LDH-hGH complex in a PAEU copolymer hydrogel, and the disordered intercalated nanostructure patterns were characterized with XRD. Aqueous solutions of the copolymer and nanobiohybrids exhibited a sol-to-gel phase transition as pH and temperature increased to physiological conditions (37°C, pH 7.4). *In vivo*, pure PAEU and its nanobiohybrid hydrogels underwent controlled degradation with LDH nanoparticles dispersed in the matrix. The biocompatibility of nanobiohybrid hydrogels was confirmed by an *in vitro* cytotoxicity test. Biodegradable, injectable, pH- and temperature-sensitive cationic PAEU hydrogels loaded with LDH-hGH had a suppressed initial burst release of hGH with a dramatically extended release period of 13 days *in vitro* and 5 days *in vivo*. Therefore, the novel biodegradable cationic nanohybrid hydrogel illustrates that LDH-hGH loaded hydrogels can be used for effective, sustained hGH delivery to improve biological efficacy and patient compliance

Acknowledgement:

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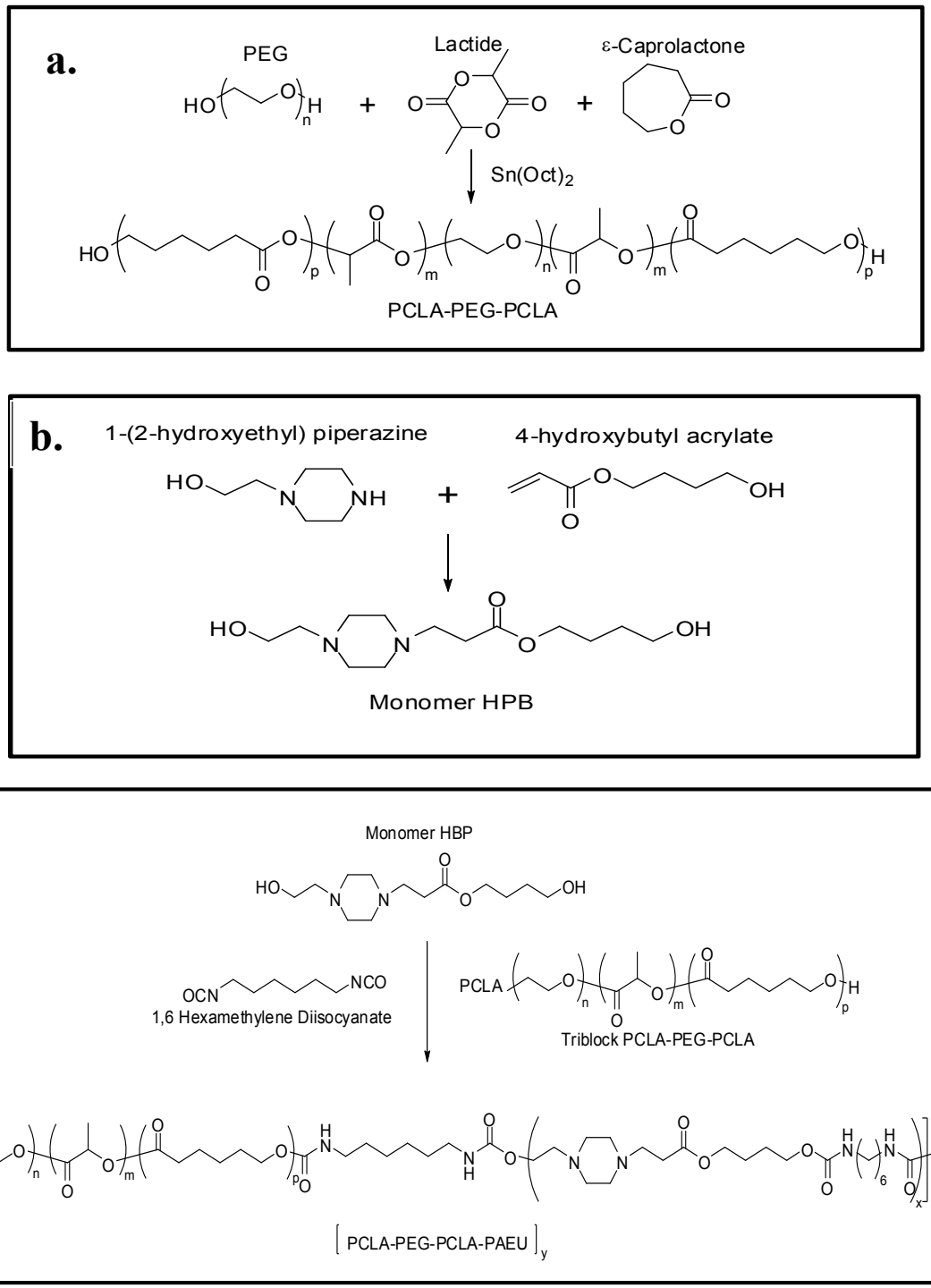

Scheme1:(a) Synthesis of triblock PCLA-PEG-PCLA, (b) monomer HPB, and (c) multiblock copolymer PCLA-PEG-PCLA-PAEU (PAEU).

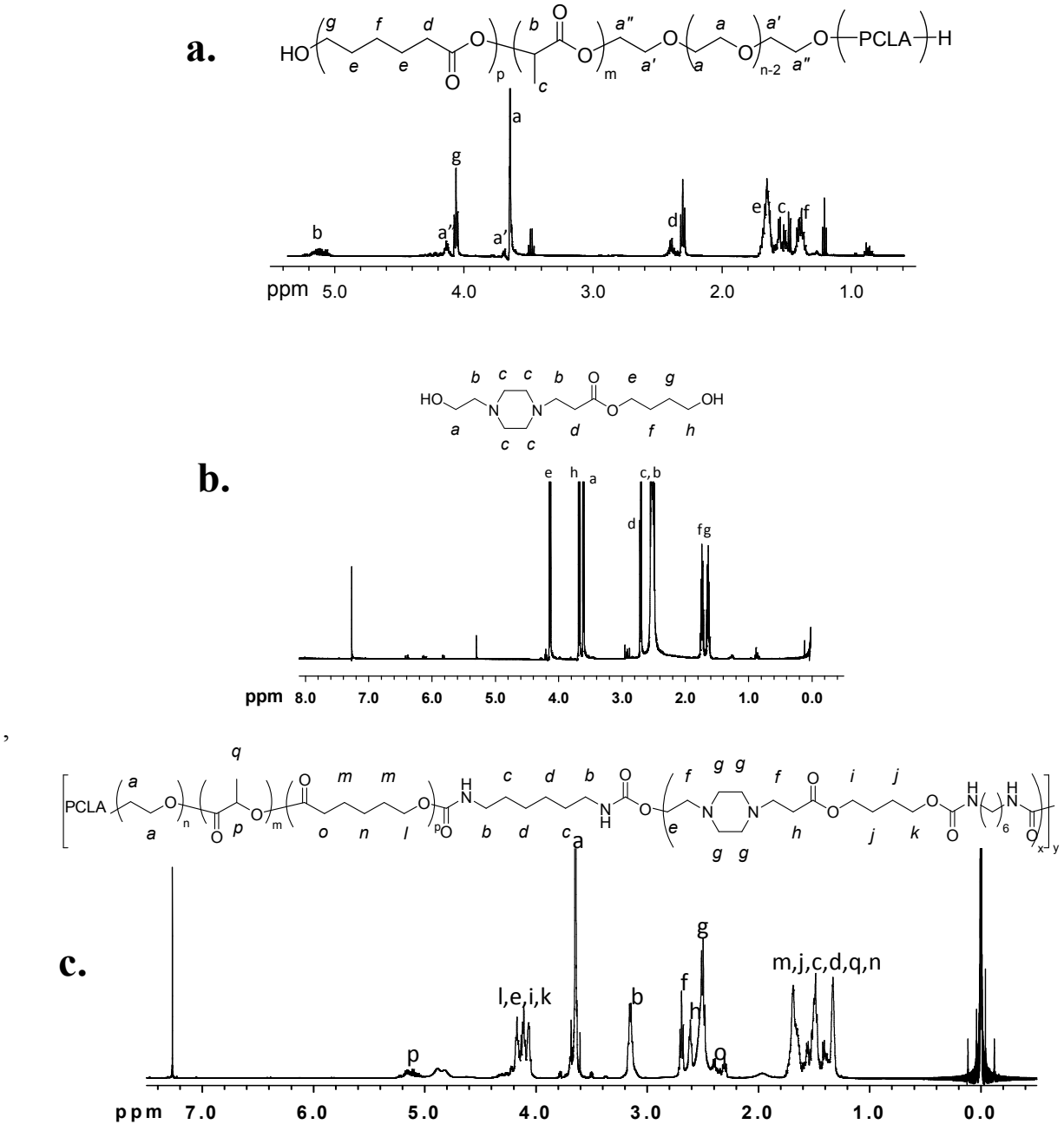

Figure 1: ^{1}H NMR spectra of the synthesized (a) PCLA-PEG-PCLA, (b) monomer HPB, and multiblock copolymer PAEU (c) in $CDCl_3$.

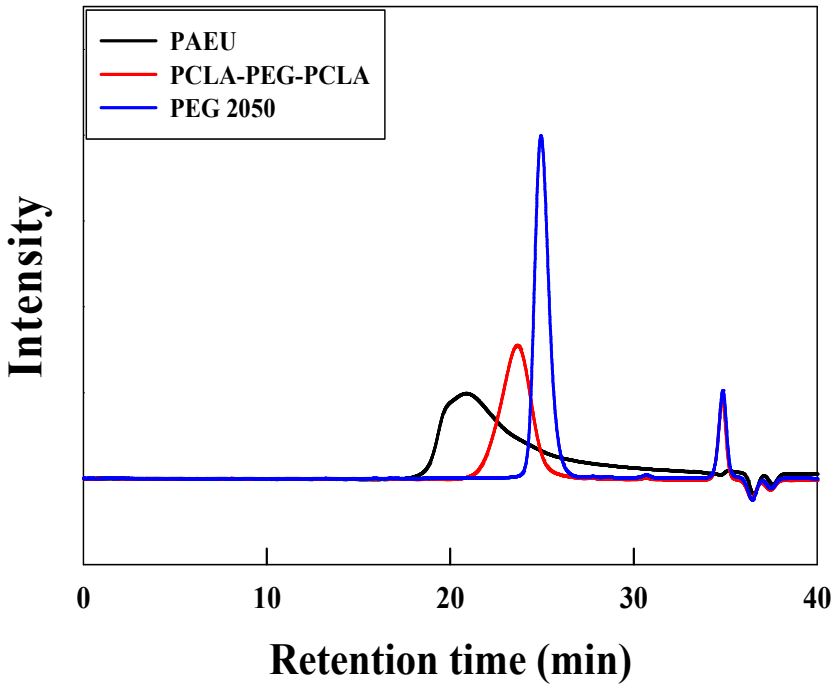

Figure 2: Gel permeation chromatography (GPC) of the synthesized PCLA-PEG-PCLA, PEG-2050, and multiblock copolymer PAEU.

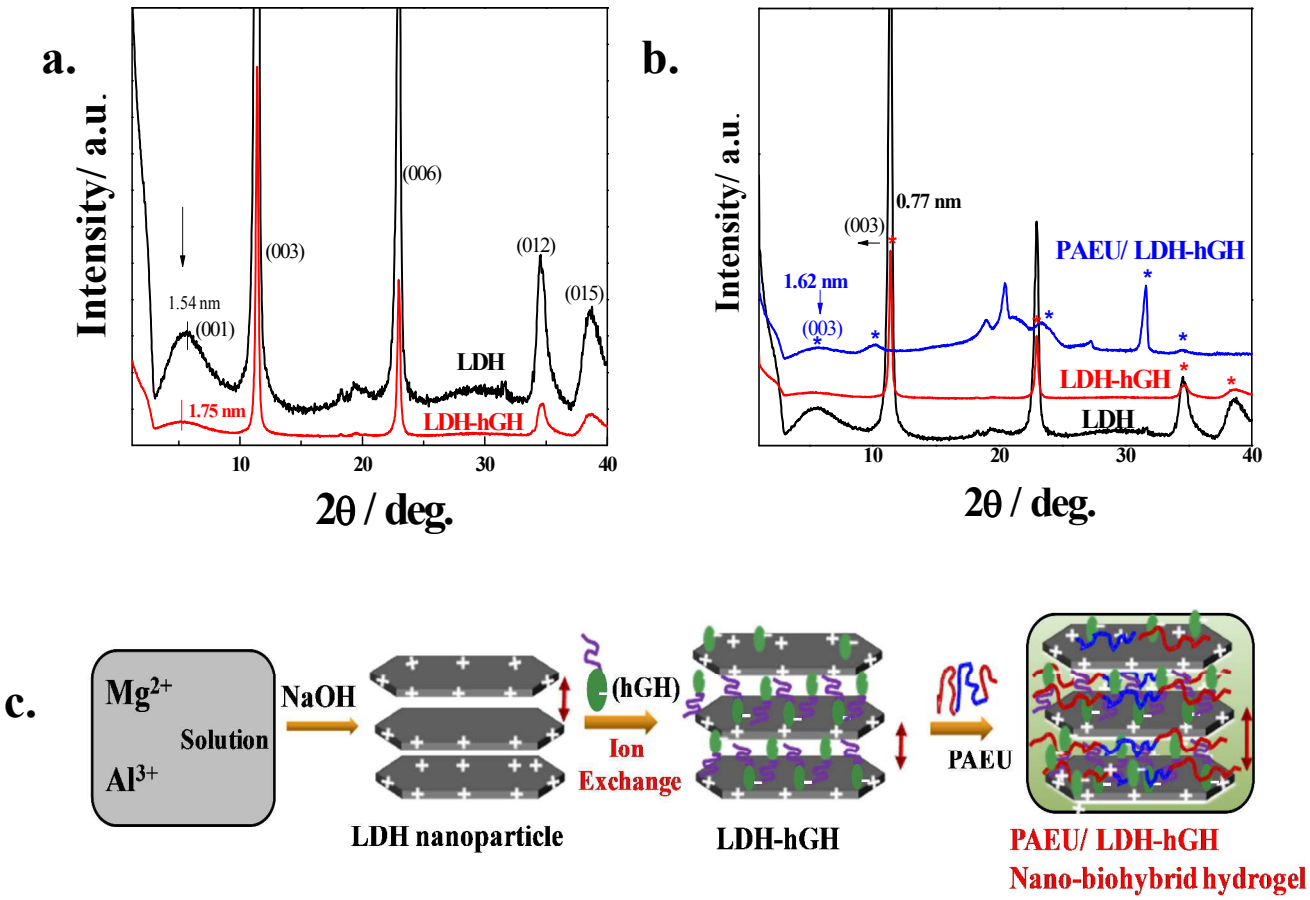

Figure 3: Wide-angle, X-ray diffraction patterns of pristine layered double hydroxide(LDH) nanoparticles, hGH-loaded LDH nanoparticles (LDH-hGH) and PAEU/LDH-hGH nanohybrid (a, b). (c) Schematic hGH loading inLDH nanoparticles and PAEU/LDH-hGH nanobiohybrid hydrogels.

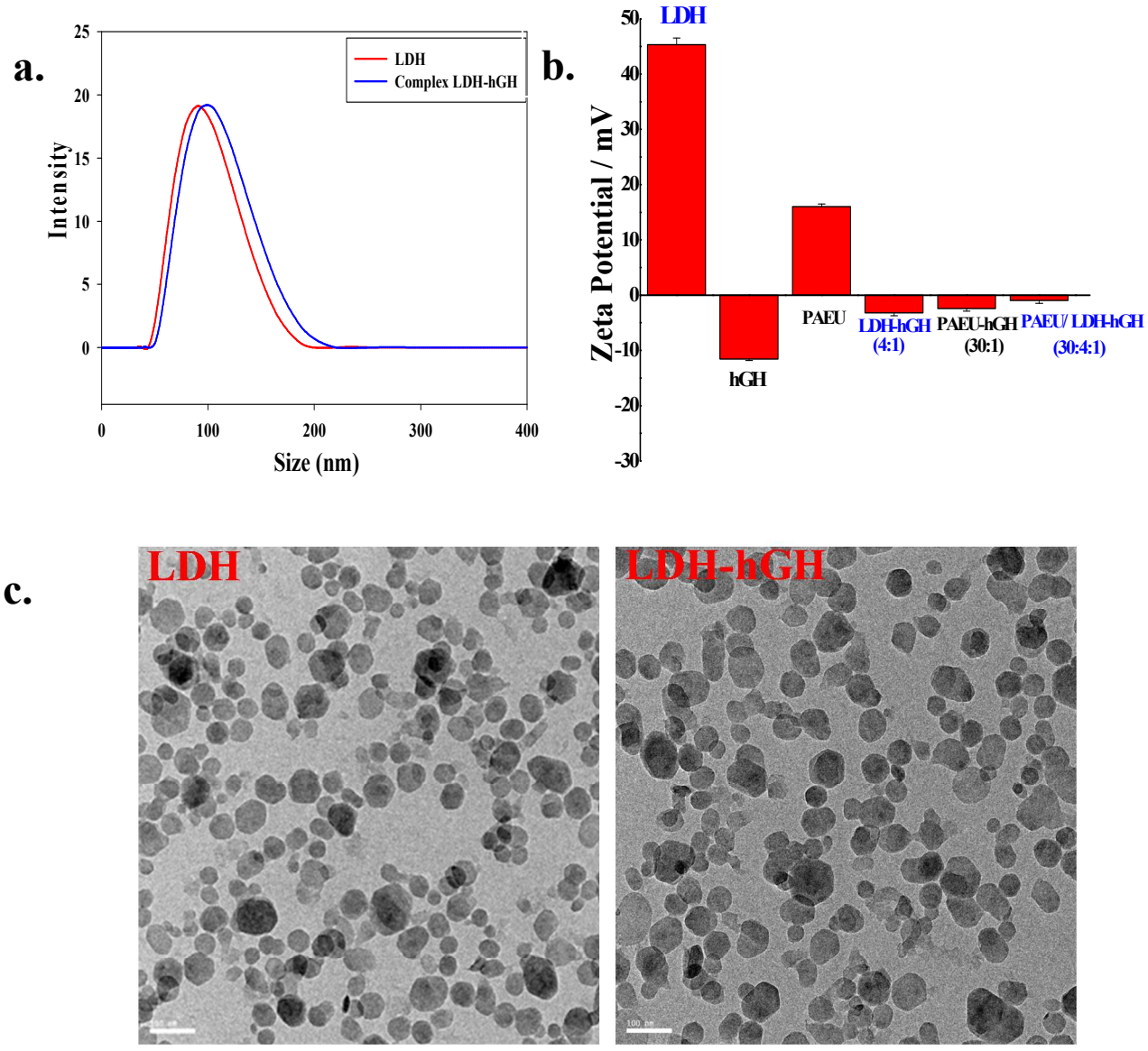

Figure 4:

Figure 4: (a) Size of LDH and LDH-hGH particles and (b) zeta potential of LDH, hGH, pristine PAEU and their corresponding LDH-hGH, PAEU-hGH, as well as PAEU/LDH-hGH nanohybrids. (c) TEM images of pristine LDH and LDH-hGH nanoparticles.

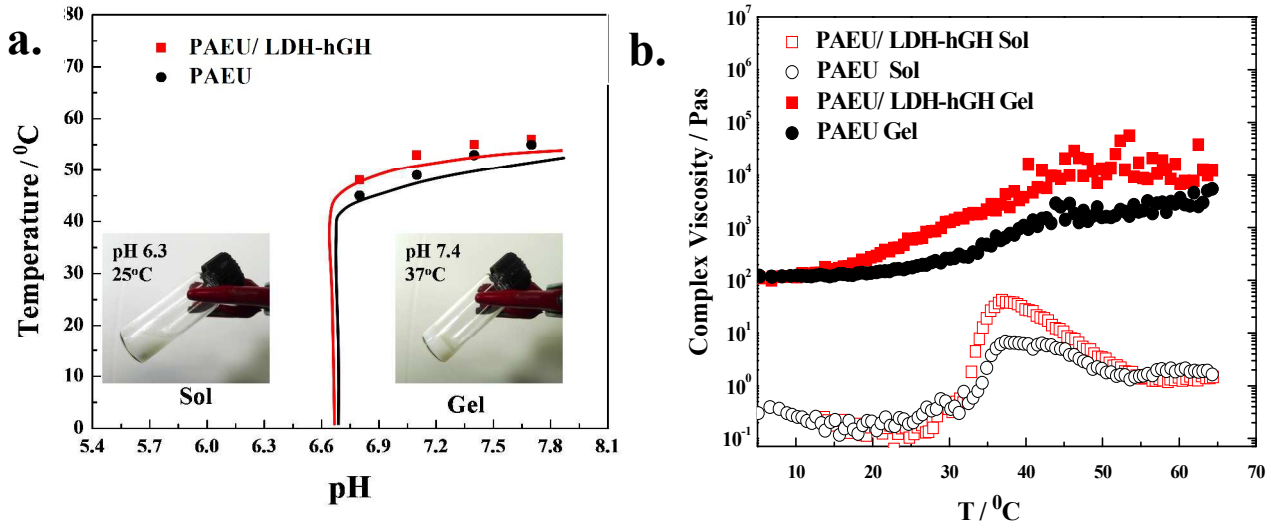

Figure 5: (a) Sol–gel phase transition diagram (b) Viscosity of 15 wt% pristine PAEU copolymer and its indicated nanohybrids solutions as a function of temperature at different pH.

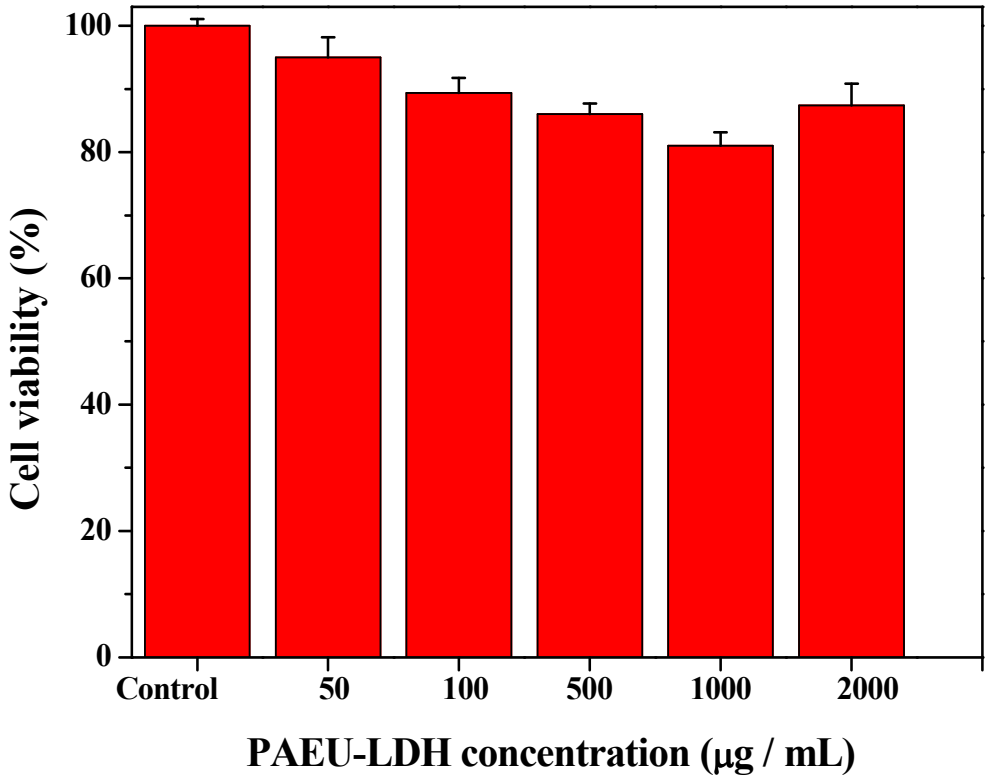

Figure 6: *In vitro* cytotoxicity of PAEU/LDH nanobiohybrid hydrogels (±SD, n = 4).

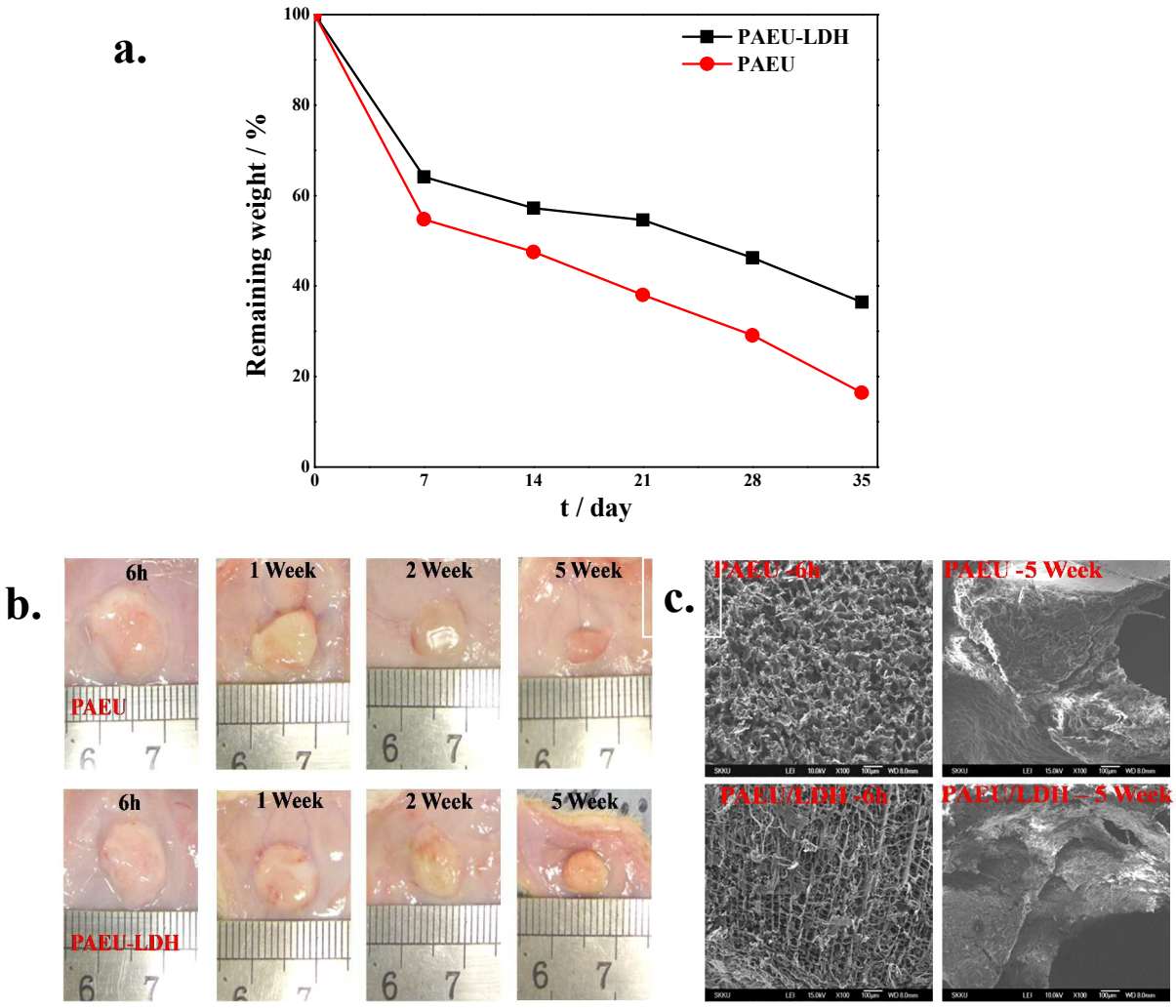

Figure 7: (a) *In vivo* degradation of PAEU and PAEU/LDH hydrogel (15 wt%, pH 6.3) determined by the mass loss method, (b) optical micrograph, and (c) SEM morphology of PAEU and PAEU/LDH hydrogels before and after degradation.

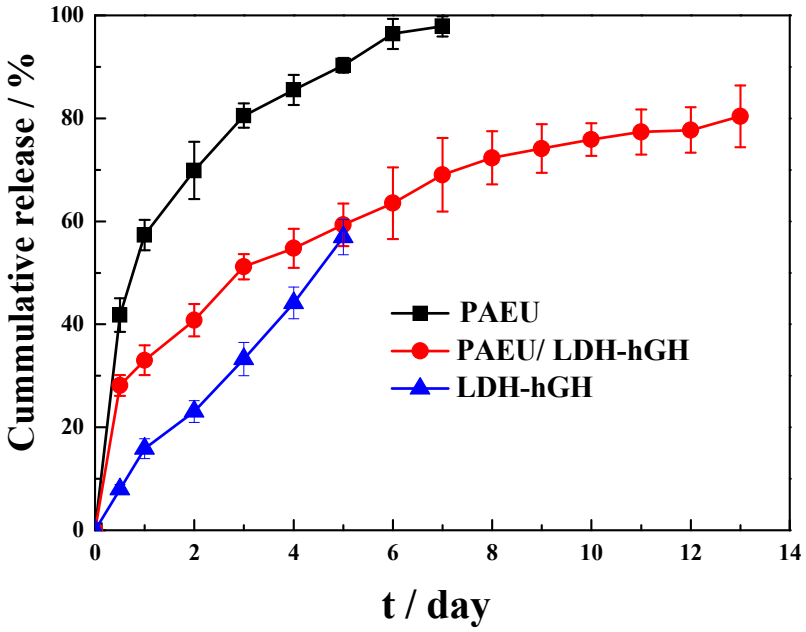

Figure 8: Cumulative *in vitro* hGH release from LDH-hGH complex, 15 wt% PAEU and PAEU/LDH-hGH nanobiohybrid hydrogels (hGH 1mg mL^{-1}) (±SD, n =3).

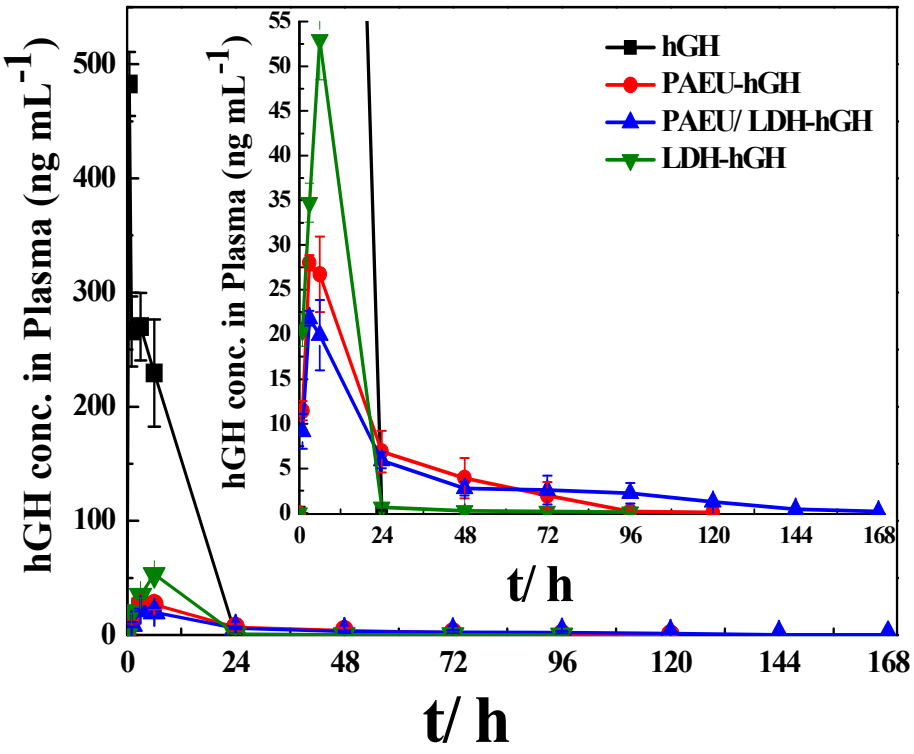

Figure 9: Human growth hormone (hGH) concentration in the serum of SD rats over time after administration of 300 µL of an hGH solution (5 mg mL^{-1}), LDH-hGH complex, hGH-loaded 15 wt% copolymer (PAEU-hGH), and PAEU/LDH-hGH nanohybrid hydrogel (hGH 5mg mL^{-1}), (±SD, n = 4).

Table of Content only

Nanostructure controlled sustain delivery of human growth hormone using injectable, biodegradable, pH / temperature responsive nanobiohybrid hydrogel

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