Biodegradable nanocomposite microparticles as drug delivering injectable cell scaffolds

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A B S T R A C T

Injectable cell scaffolds play a dual role in tissue engineering by supporting cellular functions and delivering bioactive molecules. The present study aimed at developing biodegradable nanocomposite microparticles with sustained drug delivery properties thus potentially being suitable for autologous stem cell therapy. Semi-crystalline poly(l-lactide/dl-lactide) (PLDL70) and poly(l-lactide-co-glycolide) (PLGA85) were used to prepare nanoparticles by the double emulsion method. Uniform and spherical nanoparticles were obtained at an average size of 270–300 nm. The thrombin receptor activator peptide-6 (TRAP-6) was successfully loaded in PLDL70 and PLGA85 nanoparticles. During the 30 days’ release, PLDL70 nanoparticles showed sustainable release with only 30% TRAP-6 released within the first 15 days, while almost 80% TRAP-6 was released from PLGA85 nanoparticles during the same time interval. The release mechanism was found to depend on the crystallinity and composition of the nanoparticles. Subsequently, mPEG-PLGA nanocomposite microparticles containing PLDL70 nanoparticles were produced by the ultrasonic atomization method and evaluated to successfully preserve the intrinsic particulate properties and the sustainable release profile, which was identical to that of the nanoparticles. Good cell adhesion of the human fibroblasts onto the nanocomposite microparticles was observed, indicating the desired cell biocompatibility. The presented results thus demonstrate the development of nanocomposite microparticles tailored for sustainable drug release for application as injectable cell scaffolds.

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1. Introduction

The past decades have seen the emergence of cell-based tissue engineering therapies to restore damaged or worn-out parts of the body [1]. The current strategies have utilized the autologous stem cells in combination with supportive bioresorbable matrices and bioactive molecules [2]. However, one of the biggest challenges in this approach is to create a three-dimensional structure, namely a functional cell scaffold that can promote the proliferation and differentiation of cells in order to regenerate the new tissue [3]. In the previous studies the injectable scaffolds have provided great opportunities for cell transplantation and delivery of growth enhancing components with minimal invasive surgery [4,5], thus the development of injectable scaffolds has gained great attention in the fields of autologous cell therapy and drug delivery. Various biomaterials, either natural (chitosan [6], alginate [7,8], fibrin [9]) or synthetic (polycaprolactone [10], polypropylene fumarate [11]), have been widely utilized to develop biodegradable scaffolds.

Poly(l-lactide-co-glycolide) (PLA/PLGA) polymers are a class of materials accepted by FDA for injection in this area because of their biocompatibility and biodegradability [12,13]. PLA/PLGA-based nano-/microparticles have been reported previously in numerous different applications for the delivery of biomolecules, which provided a great potential for controlled drug release [14,15]. Properties of these systems can easily be tuned using different PLA/PLGA polymers with varying compositions or molecular weights for obtaining the desired release rate, as the drug release is predominantly controlled either by diffusion or by erosion depending on the polymer compositions and molecular weight [16,17]. Furthermore, some modified copolymer types, e.g. PEGylated PLA/PLGA, offer a more hydrophilic and flexible surface, which could provide better cell affinity and biocompatibility [18]. Therefore, bioactivity of scaffolds can be achieved by loading peptides, proteins or bioactive molecules into and releasing from the PLA/PLGA-based nano-/microparticles to guide cell growth and tissue formation.

Physiologically, tissue repair is guided by the stimuli of local regulatory factors, such as different growth factors or extracellular matrix molecules, to promote specific responses to the target cells [5]. Thrombin acts as a serine proteinase to mediate the clotting at wound sites, thus it is believed to participate in regulating all stages of tissue repair [19]. A number of growth and chemotactic factors are released...
from the platelets and endothelial cells following the thrombin treatment [20]. Some studies reported that the encapsulation of thrombin-related peptides in cell scaffolds, such as the TP508 peptide, which represents the non-proteolytic receptor-binding domain of thrombin, can be beneficial for bone regeneration [21,22]. It is believed that thrombin-induced cellular activity is mediated by binding to a G-protein-coupled cell surface receptor, i.e. the protease-activated receptor PAR-1 [23]. The present study includes the use of another thrombin-related peptide, i.e. thrombin receptor activator peptide-6 (TRAP-6), as TRAP-6 was previously determined as the most potent sequence for activation of PAR-1 and mimics some of the thrombin activities [24].

It is challenging for the manufacturing process to fulfill all the requirements of scaffolds with a desirable three-dimensional morphology and mechanical structure, which could sufficiently support the cells and provide an ideal environment for cell growth. Surface and interior morphology, e.g. porosity and interconnectedness, are essential to cell adhesion and cell proliferation, but also important to the diffusion of nutrients and waste compounds [25,26]. Many techniques have been used to produce microparticles with appropriate morphology and structure. The water-in-oil-in-water (w/o/w) double emulsion-solvent evaporation method is often used to obtain PLGA microparticles with different porosities and morphologies [27,28]. This method can be modified in order to achieve a porous surface or interior structure of microparticles by using a gas foaming method or by the addition of hydrogen peroxide [27,29]. However, in an industrial perspective, the double emulsion method may to some extent have limitations with regard to up-scaling. Ultrasonic atomization is an alternative in being a gentle, versatile and reliable technique for microparticle preparation, which has been proven to encapsulate peptide or protein drugs without damaging the drug integrity and efficacy [30,31]. The major advantage of this method is that different sizes of uniform microspheres can be produced by adjusting the vibration frequency, the polymer concentration and the surface tension, so that the desirable morphology and drug encapsulation of the microspheres can be obtained [32]. As ultrasonic atomization is an easily up-scalable and industrially reliable method, it has been used to fabricate the microparticles with different encapsulation properties in this study.

Among the wide variety of different scaffolds, the common approach is to bring cells into the defect area and to support the cellular functions during the early phases of the regeneration process [33]. A simple delivery system used for cartilage repair will be the microcarriers or microparticles combined with the chondrocytes [33]. A simple delivery system used for cartilage repair will be the microcarriers or microparticles combined with the chondrocytes, which will be injected into the cartilage defects through an arthroscopic procedure and localized by a fixative precursor such as fibrin glue [34]. Ideally, the chondrocytes may be cultured in the presence of the microparticles for a period of time prior to use and optionally in addition with the adhesives such as the fibronectin, which could facilitate cell adhesion and in-growth as reported previously [35]. Thus the chondrocytes are attached onto the surface or within the structure to form the particle–cell constructs with sustained delivery of bioactive molecules capable of facilitating the cartilage formation.

The objective of this study was to develop a novel type of biodegradable nanocomposite microparticles as drug delivering injectable cell scaffolds. Two semi-crystalline polymers, namely poly(l-lactide:dl-lactide) (PLDL70) and poly(l-lactide-co-glycolide) (PLGA85), were used to prepare TRAP-6-loaded nanoparticles. The characteristics and the release mechanism of the two types of nanoparticles with different composition and crystallinity were investigated. The nanoparticles showing superior sustained drug release were further incorporated into the mPEG-PLGA microparticles to produce nanocomposite microparticles, which were examined with regard to physical characteristics, drug release property and cell adhesion.

## 2. Materials and methods

### 2.1. Materials

Poly(l-lactide:dl-lactide molar ratio 70:30) (PLDL70) and poly(l-lactide:glycolide molar ratio 85:15) (PLGA85) were provided by Purac Biochem (Gorinchem, The Netherlands) at a purity of 95%. Methyl-poly(ethylene glycol)-poly(l-lactide:glycolide molar ratio 50:50) (mPEG-PLGA), randomly arranged with 6.25% (w/w) mPEG (Mw 2 kDa), was provided by Coloplast A/S (Humlebaek, Denmark) at a purity of 99%. Thrombin receptor activator peptide-6 (TRAP-6) with a (peptide sequence SFLRN (Mw 748.9 Da, 95% purity) was purchased from CASLO Laboratory Aps. (Lyngby, Denmark). Polyvinyl alcohol (PVA) 403 was from Kuraray Chemical Co. Ltd. (Osaka, Japan). Coumarin-6 (>99.0% purity) was purchased from Sigma-Aldrich. All the other chemicals were obtained commercially at analytical grade.

### 2.2. RP-HPLC analysis of TRAP-6 peptide

The TRAP-6 concentration was analyzed by RP-HPLC (LaChrom, Hitachi Ltd., Tokyo, Japan) using an auto injector (Hitachi-Merck, LaChrom L7200). The separation of TRAP-6 was done on a C18 column (50 mm × 4.6 mm, 2.6 μm, Kinetex, Phenomenex, Allerød, Denmark) at a constant flow of 0.8 ml/min with fluorescence detection of the phenylalanine in the TRAP-6 (λex 258 nm/λem 282 nm). A mobile phase composed of solvent A (95% H2O/5% acetonitrile/0.1%TFA) and solvent B (5% H2O/95%acetonitrile/0.1%TFA) and solvent B (5% H2O/95%acetonitrile/0.1%TFA) at an A:B ratio of 85:15 was used. The limit of detection (LOD) and the limit of quantification (LOQ) were obtained to be 0.3 μg/ml (n = 3) and 1 μg/ml (n = 3) by injection of 30 μl samples. All samples were injected three times.

### 2.3. Preparation of nanoparticles

The properties of the polymers used for the preparation of nanoparticles are listed in Table 1.

PLDL70 and PLGA85 were selected due to their solubility properties in dichloromethane (DCM) and acetone. TRAP-6 loaded nanoparticles were prepared by the water-in-oil-in-water (w/o/w) double emulsion-solvent evaporation method. Briefly, 250 μl of TRAP-6 solution (0.8 mg/ml in phosphate-buffered saline (PBS), pH 7.4) was added to 500 μl of 20 mg/ml PLDL70 or PLGA85 solution in DCM. The mixture was emulsified by sonication for 90 seconds using a UP100H ultrasonic processor (Hielser Ultrasonics GmbH, Teltow, Germany) to form the primary emulsion. 1 ml of 2% (v/v) PVA in MilliQ water was immediately added to the primary emulsion and whirled mixed well for 60 seconds. The mixture was sonicated for another 60 seconds to produce the second emulsion before transfer and consequent dilution to 5 ml of 2% (v/v) aqueous PVA solution. The resulting double emulsion was stirred magnetically for 3 hours to evaporate all the organic solvent. As controls, non-loaded nanoparticles were prepared in the same way, except that 250 μl PBS buffer was added to the polymer solution instead of the TRAP-6 solution. For imaging, fluorescent nanoparticles were prepared by adding 0.25% (w/w) coumarin-6 to the DCM phase.

### Table 1

Properties of polymers used for nanoparticles.

<table>
<thead>
<tr>
<th>Polymers</th>
<th>L.G. ratio</th>
<th>Mw (kDa)</th>
<th>Melting range (°C)</th>
<th>Inherent viscosity (d/Lg)</th>
<th>Solubility in DCM</th>
<th>Solubility in Acetone</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLDL70</td>
<td>100:0</td>
<td>850</td>
<td>113.1–120.3</td>
<td>3.85</td>
<td>S</td>
<td>NS</td>
</tr>
<tr>
<td>PLGA85</td>
<td>85:15</td>
<td>560</td>
<td>126.9–144.4</td>
<td>3.11</td>
<td>S</td>
<td>NS</td>
</tr>
</tbody>
</table>

| a Lactide:glycolide ratio. |
| b Average molecular weight. |
| c Solubility reported on the data sheet from the material provider, S: soluble, NS: insoluble. |
Nanoparticles were subsequently collected by ultracentrifugation (Eppendorf Refrigerated Microcentrifuge, Hamburg, Germany) in Eppendorf tubes at 18,000 g and 4 °C for 12 min. All nanoparticles were washed twice with MilliQ water and dried in a freeze dryer (Epsilon 2–4 ISC freeze dryer, Osterode am Harz, Germany) as previously reported [36] for further usage. At least 3 batches of each type of nanoparticle were prepared.

2.4. Characterization of nanoparticles

2.4.1. Particle size and zeta potential

Particle size and size distribution of all nanoparticles were determined by dynamic light scattering, applying photon correlation spectroscopy (PCS). Freeze-dried nanoparticles were easily re-dispersed in MilliQ water applying an ultrasonic water bath and whirlmixer. The mean particle diameter (Z-average) and polydispersity index (PDI) of the nanoparticles were measured by intensity at 25 °C using a Malvern NanoZS (Malvern Instruments Ltd., Worcestershire, UK) equipped with a 633-nm laser and 173° detection optics.

The zeta potential of the nanoparticles was measured by the laser Doppler electrophoresis technique using the Malvern NanoZS. A nanosphere size standard (60 ± 2.7 nm, Duke Scientific Co., USA) and a zeta potential transfer standard (−68 ± 6.8 mV Malvern Instruments Ltd., Worcestershire, UK) were used to verify the performance of the instrument. All the samples were measured in triplicate. Malvern DTS v.5.10 software (Malvern Instruments Ltd., Worcestershire, UK) was used for data acquisition and analysis. For viscosity and refractive index, values of pure water were used.

2.4.2. Morphology

The surface morphology and size range of the nanoparticles were observed by atomic force microscopy (AFM Nanoscope V, Veeco Instruments Inc., NY, USA). Nanoparticles were re-suspended in MilliQ water and the suspensions were drop-cast onto the Si/SiO₂ substrates. Subsequently, nanoparticles were scanned by AFM in the tapping mode after the evaporation of the water. Representative images were selected from imaging 3 different samples.

2.4.3. Encapsulation efficiency

The encapsulation efficiency (EE) of TRAP-6 loaded into nanoparticles was determined by extracting the TRAP-6 from the freeze-dried nanoparticles. First, 1 mg of nanoparticles was dissolved in 200 μl of chloroform and then 0.5 ml of PBS buffer was added. The mixture was whirlmixed and kept rotating end-over-end for 2 hours to facilitate the TRAP-6 partitioning into the aqueous phase. The organic and aqueous phases were separated by ultracentrifugation at 18,000g and 4 °C for 20 min. The supernatants in the water phase were collected, and the TRAP-6 content was quantified by extracting the TRAP-6 from the freeze-dried nanoparticles. Conventional microparticles (i.e. without nanoparticles) were produced in the same way, except that 0.4 mg TRAP-6 powder was dispersed in the mPEG–PLGA solution before being pumped into the atomizer. Both the nanocomposite microparticles and the conventional microparticles were collected in ethanol kept at low temperature. The particles were then collected and dried under vacuum for 48 hours for further analysis.

2.6. Nanocomposite microparticle characterization

2.6.1. Particle size

Particle size parameters (D₅₀, D₉₀) were determined by laser diffraction using a Malvern Mastersizer 2000 (Malvern Instrument Ltd., Worcestershire, UK). Approximately 10 mg of nanocomposite microparticles or conventional microparticles were dispersed in MilliQ water and added into a small volume sample dispersion unit of the Mastersizer. The average diameter was measured by volume and plotted as particle size distributions. The span value describes the size distribution as it is a measure of the width of the volume distribution relative to the median diameter, as described in Eq. (2), where N% (N = 10, 50, 90) is the volume percentage of microspheres with diameters of Dₙ. Each of the samples (n = 3) was measured in triplicate.

\[
\text{Span} = \frac{(D_{90}\text{–}D_{10})}{D_{50}}.
\]

2.6.2. Morphology

The microparticle surface and interior morphology were determined by a scanning electron microscope (SEM, JSM-5200 JEOL Ltd., Tokyo, Japan). Microparticles were sliced to an approx. thickness of 2 mm by cryostat sectioning (CM1100, Leica Microsystems, Nussloch, Germany). Dried microparticles or sliced particle sections were spread out on a metal specimen stub and gold coated by sputter coating prior to the imaging. Representative images were selected from imaging of 2 different samples.

2.6.3. Nanoparticle distribution

Visualization of the nanoparticle distribution in the composite particles was achieved by incorporating coumarin-6 (λₓ= 464 nm/λₓm 518 nm) into the PLDL70 nanoparticles, as described in Section 2.3. Imaging was done by confocal laser scanning microscopy (CLSM) using a LSM 510 microscope equipped with an argon laser (458 and 488 nm) and a HeNe laser (543 nm) (Carl Zeiss GmBH, Jena, Germany). Data analysis was done using LSM 510 software. Images are representative of at least five sample examinations, each from two different batches.

2.7. In vitro release studies

2.7.1. In vitro TRAP-6 release from nano- and microparticles

In vitro release studies were performed with PLDL70 and PLGA85 nanoparticles, as well as with the conventional microparticles and nanocomposite microparticles. Non-loaded particles served as controls. For nanoparticles, 10 mg of non-loaded or TRAP-6-loaded nanoparticles (PLDL70 and PLGA85, respectively) were suspended in 1 ml PBS (pH 7.4) using protein low-bind eppendorf tubes; for microparticles, 100 mg non-loaded or TRAP-6-loaded conventional microparticles and nanocomposite microparticles were suspended in 1 ml PBS (pH 7.4). Both the nanoparticles and microparticles were pre-wetted in PBS using an ultrasonic water bath for 10 min. All the suspensions were incubated at 37 ± 0.1 °C in a linear shaking water
bath at 0.18 min⁻¹ (Grant Instruments Ltd., Cambridgeshire, UK). At predetermined time internals, all samples were ultracentrifuged at 18,000g for 10 min before aliquots of supernatants (125 μl) were collected. After replenishing with an equivalent volume of PBS, all release samples were re-dispersed using an ultrasonic water bath. The collected supernatants were stored at −20 °C before analysis by RP-HPLC. Each release study was done in triplicate.

In order to evaluate the physical state of the polymers and nanoparticles in the release study, original PLDL70 and PLGA85 polymers, freshly-made blank PLDL70 and PLGA85 nanoparticles, as well as nanoparticles at selected time points during the release study, were collected, freeze dried and analyzed by differential scanning calorimetry (DSC) (TA7C/1X, Perkin Elmer, Waltham, USA). Thermograms were obtained on 2–5 mg samples heated in sealed aluminum pans at a scanning rate range of 10 °C/min in a nitrogen atmosphere in the range 20–180 °C(n = 3). Pyris-Instrument managing software (Perkin Elmer, MA, USA) was used for data analysis.

2.8. In vitro cell adhesion

2.8.1. Fibroblast culture

Human fibroblasts isolated from mamma reductions by explantation were cultured in Dulbecco’s Modified Eagle Medium (DMEM) containing 10% (v/v) fetal calf serum (FCS) (D10), antibiotics (penicillin (100 U/ml) and streptomycin (100 μg/ml)). The fibroblasts were trypsinized, counted and cultured for 3 days together with the nanocomposite as well as conventional microparticles in T75 in PolyHEMA-coated flasks. The initial culturing density of the cells was 1 × 10⁴ cells/ml, and the particle concentration was 1.5 mg/ml.

2.8.2. Critical point drying

At day 3 of seeding, the fibroblasts together with the microparticles, samples were washed and prepared for imaging by an E3100 critical point dryer (Quorum Technologies Ltd., East Grinstead, UK). In brief, particles seeded with cells were washed 2 times with PBS followed by fixation for 1 and 4 hours in 1% and 3% (v/v), respectively, glutaraldehyde in MilliQ. The cell-seeded particles were subsequently dehydrated by ethanol. For this, samples were placed in a gradient of ethanol, i.e. 30%, 50%, 70%, 80%, 90%, 95% and 100% (v/v), each for 15 min. After dehydration, further substitution with CO₂-miscible liquid-amyl acetate was carried out through a series of graded baths between ethanol and amyl acetate, i.e. 75:75, 50:50, 25:75 (15 min each), and eventually the samples were immersed in 100% (v/v) amyl acetate and placed in the chamber of the critical point dryer. The chamber was filled with liquid CO₂, and subsequently amyl acetate was removed completely. Samples were immersed in liquid CO₂ for 1 hour before increasing the temperature to the critical point of CO₂ (31.5 °C, 1100 psi) at which liquid CO₂ vaporized without surface tension effects, which maintained the intact morphology of cell samples. The dried samples were sputter coated and imaged by SEM as described above.

3. Results

3.1. Characterization of nanoparticles

PLDL70 and PLGA85 nanoparticles loaded with TRAP-6 were reproducibly obtained by the double emulsion-solvent evaporation method. AFM images showed both spherical PLDL70 and PLGA85 nanoparticles with a relatively smooth surface (Fig. 1). The PLDL70 and PLGA85 nanoparticles had a mean size range of 275–300 nm with a narrow size distribution and zeta potential ranging from −35.1 to −40.5 mV. Compared to the non-loaded nanoparticles, TRAP-6 loading did not significantly affect the particle size and zeta potential. Coumarin-6 loaded PLDL70 nanoparticles for the confocal imaging showed identical size distribution (data not shown). The encapsulation efficiency was improved by process optimization from initial 5% to 15.4% and 10.3% for PLDL70 and PLGA85 nanoparticles, respectively. The characteristics of nanoparticles are summarized in Table 2.

3.2. In vitro TRAP-6 release from nanoparticles

Accumulative release of TRAP-6 from PLDL70 and PLGA85 nanoparticles was monitored over 30 days. PLDL70 nanoparticles displayed a sustained release profile (Fig. 2a), which can be divided into three phases: (i) initial burst release of 10% TRAP-6 during the first day; (ii) slow sustained release of additional 20% peptide over 15 days; (iii) faster release of an additional 50% of the encapsulated TRAP-6 until the 30th day. After the 30 days, over 20% TRAP-6 was recovered from the remaining PLDL70 nanoparticles, providing a mass balance of 95 ± 5%. In contrast, PLGA85 nanoparticles showed a rapid release with almost 50% initial burst release in the first day and 80% released over 15 days. Hardy any TRAP-6 was recovered from the PLGA85 nanoparticles after 30 days, which shows the complete release of TRAP-6 from PLGA85 nanoparticles. As a consequence of the release profiles, PLDL70 nanoparticles with higher sustained release were selected for the further formulation of nanocomposite microparticle.

In order to explain the different release profiles of these two types of nanoparticles, DSC thermograms of the pure polymer and nanoparticles on day 0, day 1, day 10 and day 18 of the release experiment were analyzed. The samples for the DSC corresponded to different phases of the release profile as indicated in Fig. 2. Overall, the DSC data demonstrated differences in the phase transition observed between the polymer and freshly-made nanoparticles, as well as among the nanoparticles from the release experiment (Fig. 3). The glass transition temperature (Tg) of PLDL 70 nanoparticles was observed at approximately 40 °C and increased slightly during the release experiment, yet, the Tg of PLGA 85 nanoparticles did not seem to increase until the 18th day. The PLDL70 polymer showed two melting peaks (Fig. 3a), in which the higher peak at 113–123 °C was identical to the melting point (Table 1), while the lower peak at 60–65 °C probably indicated the melting point of the existing L-lactide oligomers in the PLDL70 polymer [37], which may be the residuals from the polymer production or a degradation product formed during the storage. However, there was no melting peak in the formulated PLDL70 nanoparticles (Fig. 3b), which indicates that the PLDL70 in the nanoparticles was in the amorphous state caused by the preparation process. But on day 1 and day 10 after initiation of the release study, the peak at 60–65 °C reappeared, probably indicating certain crystalline growth (Figs. 3c and d). At day 18, the peak at 60–65 °C disappeared (Fig. 3e), which most likely indicates an amorphous state of PLDL70 nanoparticles due to the absence of any melting peak. Similar to the PLDL70 polymer, the PLGA85 polymer also demonstrated a semi-crystalline structure with two melting peaks, which were the melting points of PLGA85 at 126–144 °C (Table 1) and l-lactide oligomers at 60–65 °C, respectively (Fig. 3f). The two peaks were still present in the thermograms of the freshly-made PLGA85 nanoparticles, i.e. at day 0 (Fig. 3g), demonstrating that, unlike the PLDL70 nanoparticles, the crystalline structure was not highly affected by the preparation process. The PLGA85 nanoparticles also showed an increase of the peak at 60–65 °C during the release between day 1 and day 10 (Fig. 3h and i), but it was not observed at day 18 which is similar to that of PLDL70 nanoparticles. In all samples of PLGA85, including the polymer and nanoparticles, the higher melting peak was observed (Fig. 3f–j), indicating that the PLGA nanoparticles were in partially crystalline structure at all times during the release up to 18 days.

3.3. Characterization of nanocomposite microparticles

According to the release profile, TRAP-6-loaded PLDL70 nanoparticles were selected and incorporated into mPEG–PLGA microparticles to form nanocomposite microparticles. As shown in Table 3, PLDL70/mPEG–PLGA
nanocomposite microparticles showed a mean particle size of 76.1 μm. Compared to the conventional microparticles with a mean particle size of 82.6 μm, the inclusion of nanoparticles did not significantly change the particle size of composites (P = 0.3). The size range of the nanocomposite microparticles prepared by ultrasonic atomization was in an acceptable dimension (80–100 μm) for use as the injectable cell scaffolds [38].

SEM images showed the spherical nanocomposite microparticles with uniform size and porous surface (Fig. 4a and b). The conventional microparticles displayed similar size and surface morphology (data not shown); however, the interior structure demonstrated relatively more hollow structure compared to the nanocomposite (Fig. 4c and d).

Fig. 5 shows the distribution of PLDL70 nanoparticles labeled with coumarin-6 in the composite microparticles. Horizontal scanning of the nanocomposite microparticles in 4 μm steps demonstrated that the PLDL70 nanoparticles (in green) were observed primarily on the outer layers of the microparticles, most likely due to the much denser structures of the outer layers which contained more nanoparticles. Leakage of coumarin-6 from the nanoparticles during the processing of the nanocomposite particles was checked for and observed not to have occurred.

3.4. Sustained release from nanocomposite microparticles

In order to investigate the release properties of PLDL70/mPEG-PLGA nanocomposite microparticles, TRAP-6 release from the nanocomposite microparticles was studied over 30 days in vitro (Fig. 6). Compared to the conventional microparticles, which resulted in more than 70% burst release within the first day, the nanocomposite nanoparticles showed a prolonged release profile similar to that of PLDL70 nanoparticles, indicating that the properties of the nanoparticles were not affected by the process of ultrasonic atomization. The results show that the nanocomposite microparticles have the ability to preserve the sustained release profile of the PLDL70 nanoparticles [39,40].

**Table 2**

<table>
<thead>
<tr>
<th>Polymers</th>
<th>Loaded Drug</th>
<th>Diameter (nm)</th>
<th>PDII</th>
<th>Zeta potential (mV)</th>
<th>EE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLDL70</td>
<td>TRAP-6</td>
<td>286.4 ± 3.5</td>
<td>0.186 ± 0.011</td>
<td>−37.2 ± 2.0</td>
<td>15.4 ± 4.5</td>
</tr>
<tr>
<td>PLGA85</td>
<td>TRAP-6</td>
<td>292.2 ± 9.6</td>
<td>0.131 ± 0.012</td>
<td>−40.5 ± 1.2</td>
<td>10.3 ± 3.6</td>
</tr>
</tbody>
</table>

Samples represent the mean of three determinations ± standard deviation.

* Polydispersity index.

* Encapsulation efficiency.

**Fig. 1.** Atomic force microscopy (AFM) images of (a) PLDL70 nanoparticles, scale bar = 500 nm and (b) PLGA85 nanoparticles, scale bar = 250 nm.
many studies, the development of composite scaffolds has been in focus for demonstrating superior properties with regard to cell affinity and controlled drug release, e.g., for bone or cartilage regeneration [41,42]. With the aim of developing injectable cell scaffolds with sustained drug delivery function, nanocomposite microparticles were tailor-designed by incorporating TRAP-6-loaded PLDL70 nanoparticles into mPEG–PLGA microparticles. This study displayed the characterization and careful selection of the appropriate nanoparticles as the drug delivery system, and subsequently the incorporation into composite microparticles as cell scaffolds with controlled release properties.

Two biodegradable polymers with crystalline structure, PLDL70 and PLGA85 were chosen for preparation of the nanoparticles based on their insolubility in acetone, which is essential to the following step for successful preparation of the microparticulate cell scaffold. Nanoparticles were prepared by the double emulsion-solvent evaporation method, which is well known to be applicable for reproducibly obtaining fairly homogenous nanospheres with an average size of 200–250 nm [36,43,44]. PLDL70 and PLGA85 nanoparticles showed good colloidal stability without aggregation and easy re-dispersion after freeze drying. However, the challenge when using the double emulsion method is the resulting low encapsulation efficiency of hydrophilic drugs to the PLGA polymer matrices, which is largely due to the low affinity of such drugs to the hydrophobic polymers. Some studies demonstrated that the loading of hydrophilic drugs can be improved by increasing the partitioning of the drug to the organic phase, for instance by tuning the pH of aqueous phase [45], complexation with phospholipids [46] or ion-pairing agents [47,48]. Other studies also demonstrated that optimizing the processing parameters via a factorial design can help to achieve higher drug loading [36]. As described [49], the encapsulation efficiency of water-soluble drug was mostly around 5%–20%, and in the present study the optimizations increased the TRAP-6 encapsulation from around 5%–15%. Further enhancement of the encapsulation efficiency may be achievable, but it was not the primary aim of the present study.

Even though the PLDL70 and PLGA85 nanoparticles displayed a similar surface morphology and encapsulation efficiency, the release study showed different release profiles, which is probably due to the differences in crystallinity and composition between PLDL70 and PLGA85. DSC thermal analysis data demonstrated changes in the phase transition before and after the production of nanoparticles, as well as during the release period. Considering the originally semi-crystalline structure of both PLDL70 and PLGA85 polymers, freshly-made PLDL70 nanoparticles (at day 0) seemed to be in the amorphous structure, while PLGA85 nanoparticles were still partially in the crystalline state. During the first 10 days of the release study, the crystallization process of L-lactide oligomers occurred in both PLDL70 and PLGA85 nanoparticles, probably due to the exposure to moisture and the temperature of the incubation condition [50]. The DSC analysis supported the release profiles, indicating that the release from PLDL70 nanoparticles was both diffusion and erosion controlled, especially as seen from the triphasic release of TRAP-6 from PLDL70 nanoparticles [51]. In the first 15 days’ lag time, the PLDL70 nanoparticles displayed a much lower initial burst release than PLGA85 nanoparticles. This was probably because of the better dispersion of drug in the amorphous polymer matrix with good drug–polymer interactions, which consequently decreased the diffusion rate from PLDL70 nanoparticles, while the zero-order release starting from day 15 was most likely dominated by the initiation of surface erosion [52], which was confirmed by the DSC curve showing the crystalline structure changed to amorphous on day 18. The mechanism of the release from the PLGA85 nanoparticles is also expected to be a result of diffusion. The existing crystalline structure in PLGA85 nanoparticles hampered the dispersion of drug in the matrices, which consequently resulted in rapid initial diffusion from the polymer matrices. Previous studies also reported similar findings.

### Table 3

<table>
<thead>
<tr>
<th>Microparticles</th>
<th>Loaded nanoparticles (w/w)</th>
<th>(D_{\text{ave}}) (nm)</th>
<th>Span value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conventional</td>
<td>82.5 ± 8.5</td>
<td>1.7 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>Nanocomposite</td>
<td>76.1 ± 9.4</td>
<td>1.5 ± 0.2</td>
<td></td>
</tr>
</tbody>
</table>

Samples represent the mean of three determinations ± standard deviation.
that crystalline particles resulted in a much higher initial burst release than the amorphous ones [50,53]. In addition, the slight increases in the Tg of both nanoparticles were due to the physical aging of the polymer during the storage time [54], during which the polymer chains approached the equilibrium state and the cohesional entanglements were formed by chain relaxation; therefore more energy is needed to disengage the tangled chain structures, which resulted in the Tg shifting to a higher temperature [55].

Besides the crystallinity, the composition of the polymers is also likely to be related to the drug release profiles. Many studies have shown that the L:G ratio of PLGA polymers strongly influences the drug release [56,57]. The rather fast initial burst release from PLGA85 nanoparticles could also be caused by the more hydrophilic PLGA compared to PLA in the PLDL70. The more hydrophilic polymer has a higher tendency to take up the water and hydrate the polymer matrices, which would relax the polymer chain and increase the diffusion of drug. Furthermore, the influence would be more significant on the release of a drug with more hydrophilic property, like TRAP-6 has compared to a hydrophobic drug [58], as the hydrophilic drug has a higher affinity to the water than to the polymer matrix.

The sustained drug release was considered an imperative criterion for selecting PLDL70 nanoparticles to be incorporated into mPEG-PLGA microparticles. For the purpose of obtaining successful composite scaffolds, many reports have been published on blends of various multifunctional materials to enhance the properties of the scaffolds [41,59], and on imbedding nano-structures into the scaffolds to obtain more controllable structures and properties [60,61].
Novel nanocomposite microparticles demonstrating sustained release of a bioactive drug over 30 days have been developed for potential use as injectable cell scaffolds. The drug release mechanism from two types of nanoparticles based on PLDL70 and PLGA85 was shown to be related to the polymer crystallinity and composition. The PLDL70 nanoparticles showed a more prolonged release and were composited with mPEG-PLGA microparticles to form nanocomposite microparticles. The composites thus produced demonstrated a similarly sustainable release as the nanoparticles. In addition, good cell adhesion onto the nanocomposite microparticles further contributed to the potential for use as injectable cell scaffolds. These results provide a novel method for obtaining injectable composites with tailored drug delivery properties. Future work will be able to apply this method for the delivery of other bioactive substances to achieve better in vitro and in vivo effects for autologous stem cell therapy.

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