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Multilayered drug delivery coatings composed of daidzein-loaded PHBV microspheres embedded in a biodegradable polymer matrix by electrophoretic deposition†

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The development of drug delivery coating systems for local and long-term drug release is gaining increasing interest especially to functionalize bioinert implants with osseointegration and antibacterial properties. In this study, a biodegradable drug delivery coating platform consisting of drug-loaded PHBV microspheres embedded in an alginate–PVA matrix was fabricated by a one-step electrophoretic deposition (EPD) process. Layer by layer (LbL) deposition was exploited to generate chitosan–alginate multilayers on the EPD-produced coating to enlarge the diffusional barrier around the microspheres for controlled drug release. Daidzein, selected as a model drug due to its anti-osteoporosis properties, was pre-encapsulated in PHBV microspheres. The parameters for microsphere fabrication were optimized by an orthogonal design approach. The loading efficiency of daidzein in both the microspheres and in the deposited coatings was adjusted by varying the processing parameters during microsphere fabrication and the EPD process. The degradation of the deposited multilayers was investigated in PBS for up to 14 days. The degradation rate, surface roughness and wettability, as well as adhesion strength of the coatings during degradation were evaluated by applying a range of techniques. A controlled and sustained daidzein release was detected from both free microspheres and microsphere-containing coatings. Finally cytotoxicity and stimulatory effects of daidzein or daidzein-loaded coatings, on both MC3T3-E1 and RAW264.7 cell lines, were studied to validate the potential of the developed coatings for orthopedic applications.

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1. Introduction

Modification of orthopedic implants with bioactive coatings is being intensively investigated with the aim of reducing the risk of implant failure associated with weak bone-to-implant contact

and/or due to infections.^{1,2} In particular, polymer based coatings can serve as drug delivery platforms, releasing biomolecules (antibiotics, growth factors, proteins, peptides, genes) preloaded into the coating matrix, to convert conventional implants into biologically active components.^{3,4} In comparison with conventional oral or injectable therapies usually associated with side effects, such coating systems should be able to release drug molecules locally and in a controlled manner for prolonged periods.^{3,5} Local drug release provides the possibility of an efficient dosage at the defect site while avoiding undesired systemic effects. Furthermore, the coating materials, mainly constituted of water-soluble or biodegradable polymers, could be degraded gradually under physiological conditions. In many drug delivery systems aiming at controlled release, a large amount of drug molecules is initially released in a short time after soaking in the release medium, which is referred to as ‘burst release’.⁶ Burst release will lead to high drug loss (high drug concentration) potentially causing locally toxic effects and reducing the lifetime of drug delivery devices. Therefore, methods need to be developed to minimize the unexpected burst release for long-term treatment with required drug dosages.

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Encapsulating drugs in polymer microspheres is an effective approach to reduce burst release and to provide sustained and controlled drug release profiles.^{7,8} For example, vancomycin pre-encapsulated in poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) microspheres exhibited a lower burst release and sustained drug release for a much longer period than direct loading in PHBV films.⁹ PHBV is a copolymer of PHB and PHV, and has been widely used for producing drug loaded microspheres due to its good biocompatibility and tailorable degradability.¹⁰ Moreover, PHBV does not usually produce harmful acidic degradation products.¹¹ These advantages make PHBV microspheres attractive drug carriers for biomedical applications.

During our previous investigation of PHBV-containing drug delivery coatings,¹² it was confirmed that antibiotics were able to be preloaded into PHBV microspheres, and then embedded in an alginate matrix for antibacterial purpose. It is expected that this coating platform is versatile to deliver not only antibiotics but also other cellular stimulating molecules with hydrophilic or hydrophobic character, in a controlled manner, for different applications. In this study, an improved PHBV microsphere-alginate coating system was developed for the delivery of daidzein, a natural isoflavone which is being considered to treat osteoporosis, as discussed previously.^{13–15} Daidzein can bind to the α and β estrogen receptors, which cause estrogen like effects, and thus is able to counteract the discrepancy between bone growth and resorption in osteoporotic bone.¹⁶ In addition, daidzein has an anabolic effect on osteoblast cells *via* a genomic pathway operating through estrogen receptors and gene expression mechanisms.^{15,17} However, the oral bioavailability of daidzein is very poor,¹³ which explains the need for developing vehicles for its local release with the potential to increase its bioavailability. It is therefore expected that a daidzein-loaded microsphere coating system will possess the potential to stimulate bone related cells for enhanced osseointegration.

Taking advantage of the controllable drug release performance from PHBV microspheres¹⁸ and the simplicity of the electrophoretic deposition (EPD) technique to produce polymer coatings on metallic substrates,^{19,20} PHBV microspheres were able to be stabilized into a polymer coating, and a minimized and prolonged drug release rate could be expected. Combination of PHBV microspheres and biopolymers was investigated by EPD at first. Additionally, layer by layer (LbL) deposition was introduced to produce additional barrier layers consisting of alginate and chitosan coatings, to further suppress potential burst drug release. The loading efficiency of daidzein in both microspheres and coatings was investigated, and the drug release profile, affected by a combination of the degradation rate and coating structure, was characterized for a comprehensive understanding of the drug release kinetics from the coatings.

2. Experimental

2.1 Materials

Sodium alginate and chitosan powders were purchased from Sigma Aldrich. PHBV (12 wt% PHV) was supplied by Goodfellow (Huntingdon, UK). Polyvinyl alcohol (PVA) (MW ~ 30 000) was

Fig. 1 Schematic representation of the oil-in-water (O/W) single emulsion solvent evaporation method for preparing daidzein loaded PHBV microspheres.

acquired from Merck (Darmstadt, Germany). Daidzein was purchased from Cayman Chemical (Ann Arbor, MI, USA). All the other chemicals for microsphere production and phosphate buffered saline (PBS) preparation were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Preparation of daidzein-loaded PHBV microspheres

An oil-in-water (O/W) single emulsion method was used to fabricate daidzein-loaded PHBV microspheres, as schematically shown in Fig. 1. Firstly, an orthogonal design was applied to optimize the main processing parameters (see Table S1, ESI[†]), *i.e.*, the stirring rate, PHBV concentration and PVA concentration. The optimized parameters, which are a PHBV concentration of 3% w/v, a PVA concentration of 2% w/v, and a stirring rate of 11 000 rpm, were chosen according to the particle size distribution, shape and yield of microspheres. Briefly, 5 wt% or 10 wt% daidzein was added into 3 ml of 3% w/v PHBV solution (O phase) in which PHBV was dissolved in dichloromethane (DCM) (Merck, Darmstadt, Germany) (samples named as 5% and 10%). The mixture solution was immediately added into 75 ml of 2% w/v aqueous PVA solution and emulsified using a homogenizer (T18, IKA, Germany) at 11 000 rpm for 3 min. The resultant O/W single emulsion was then stirred at 600 rpm for 3 h on a magnetic stirrer to evaporate the DCM. Microspheres were collected by centrifugation (Centrifuge 5430R, Eppendorf, Germany) at 4800 rpm for 4 min, rinsed three times in deionized water, and lyophilized in a freeze dryer (Alpha 1–2 LDplus, Martin Christ, Germany). The microspheres were then stored in a freezer for further use.

The loading degree, expressed as $[(\text{mass of loaded drug}/\text{mass of drug loaded microspheres}) \times 100\%]$, of daidzein-loaded PHBV microspheres was determined by immersing them into DCM to dissolve the polymer matrix and to release all the drug content. The encapsulation efficiency was calculated as $[(\text{the actual amount of loaded drug}/\text{theoretical amount of loaded drug}) \times 100\%]$. The amount of daidzein was quantified using a UV-vis spectrophotometer (Specord 40, analytic Jena) at a wavelength of 252 nm.

2.3 Preparation of EPD suspension

A PVA aqueous solution (20 g l⁻¹) was firstly prepared by dissolving PVA particles into deionized water with vigorous stirring at 80 °C. After cooling down the PVA solution, alginate powder was gently added to the PVA solution and the mixture was magnetically stirred for 5 min followed by a 10 min sonication

step until a transparent solution was obtained (ultrasonic bath, Bandelin Sonorex, Germany). Daidzein loaded microspheres were added into the alginate–PVA solution and thoroughly dispersed using an ultrasonic probe (Branson Sonifier[®] S250D, Emerson, USA, time: 10 s, amplitude: 30%). In order to suppress the electrolysis of water, a small amount of ethanol was added into the alginate–PVA–PHBV microsphere suspension. The final concentrations of alginate and PVA were 2 and 20 g l⁻¹, respectively. The concentration of daidzein loaded PHBV varied from 0.5 to 2 g l⁻¹.

2.4 EPD process

EPD was carried out in an EPD cell containing two parallel 316L stainless steel (SS) electrodes fixed at a distance of 10 mm. The electrodes were immersed in the prepared suspension with a deposition area of 15 × 20 mm². Due to the fact that alginate is negatively charged in an aqueous solvent and the addition of PVA was not expected to affect this behavior,²¹ alginate deposition was expected to occur on the positive electrode. In addition, PHBV microspheres in suspension were able to be co-deposited with alginate as previously discussed.¹² Various applied voltages (5–45 V) and deposition times (0.5–5 min) were investigated in preliminary tests, and the optimal voltage and deposition time were fixed at 17 V and 1 min, respectively. After deposition, the electrodes were slowly withdrawn from the EPD suspension, and coated electrodes were dried in the horizontal position at room temperature.

2.5 LbL deposition

LbL deposition was applied in this study to generate additional polymer layers on top of the EPD produced coatings. A polyelectrolyte multilayer structure consisting of alternative deposition of chitosan and alginate was prepared and the corresponding chitosan (positively charged) and alginate (negatively charged) solutions were prepared according to the literature.^{22,23} Briefly, an acetate buffer was prepared at pH 3 or 5 using appropriate volumes of 0.1 M acetic acid and 0.1 M sodium acetate. 5 mg ml⁻¹ of alginate was prepared in the low pH buffer (pH = 3) and 1 mg l⁻¹ of chitosan was prepared in the high pH buffer (pH = 5). The content of PHBV microspheres (10 wt% of daidzein loading) in the EPD coating was fixed at 2 g l⁻¹. The coated substrate was firstly dipped in the chitosan solution for 15 s and then slightly rinsed with the flowing buffer solvent (pH 5) for 15 s. Then the substrate was dipped into the alginate solution for another 15 s and then slightly rinsed with the flowing buffer solvent (pH 3) for 15 s. After each dipping process the coated substrate was dried in the horizontal position at room temperature. The final multilayered structure achieved is schematically described in Fig. 2. It was expected that the different layers are tightly connected due to the electrostatic attraction of alginate (–) and chitosan (+).

2.6 Characterization methods

The surface morphology of microspheres and coatings was observed using scanning electron microscopy (SEM, Ultra Plus and Auriga, Zeiss, Germany). Particle size distribution was

Fig. 2 Schematic diagram showing the sample code and arrangement of the multilayer structure of the deposited coatings, A, B, and C stand for EPD coating, EPD + chitosan coating, and EPD + chitosan + alginate coating, respectively.

determined using Image J (NIH, USA). Three SEM images were analyzed, and at least 100 microspheres were counted for each image. 3D topographical features of the deposited coatings were quantitatively assessed by confocal microscopy (Leica DCM 3D, Germany). An objective EPI 20X-L with a 1 μm spatial pinhole was used to detect an image area of 4 × 4 mm². The measurement also allowed us to assess the root mean square height, corresponding to the standard deviation of the height distribution, indicating the roughness distribution on the surface. The wettability/porosity of the incubated samples was evaluated by water contact angle measurement (DSA30, Krüss GmbH, Germany). Five parallel tests were performed for each coating condition. The coating weight (mg cm⁻²) as a function of incubation time in PBS was recorded using an analytical balance (accuracy of 0.1 mg) to evaluate the degradation behavior of the prepared multilayer coatings. Three samples per each time point were recorded. The adhesion strength of the deposited coatings as a function of microsphere addition or incubation time in PBS was measured using a scratch tester (CSM instruments, Revetest[®], Switzerland). A scratch length of 5 mm, a scratch speed of 10 mm min⁻¹ and a linearly increased load in the range of 0.09–20 N were used for the measurement. Due to the insignificant response from acoustic emission signals, the load at which cracks were first observed to occur along the scratch track was determined as the critical load, which is achieved with the help of a combined optical microscope. At least seven parallel tests were performed for each coating condition.

2.7 Drug release study

Due to the poor solubility of daidzein molecules in aqueous solution, a mixture solution consisting of 75 vol% PBS and 25 vol% ethanol was used for the daidzein release study in order to meet sink conditions, as previously explained in the literature.^{18,24} Both microspheres (2 mg prepared with 5 and 10 wt% of daidzein) and coatings were soaked into 1 ml medium in a cellulose dialysis bag (Spectra/Por[®] 1, molecular weight cut-off 6000 to 8000, Carl Roth, Germany). Then the dialysis bag was put into a plastic tube and filled with another 4 ml of releasing medium. The soaked samples were located in an incubator at 37 °C at a shaking speed of 90 rpm. After the predetermined incubation time, 1 ml of solution was taken out for measurement and 1 ml of fresh medium was added for continuous

drug release. The concentration of daidzein was measured using a UV-vis spectrophotometer, and the cumulative release of daidzein was calculated. The drug release study was performed in triplicate. Higuchi and Peppas models, as represented by eqn (1) and (2), were used to analyze the drug release kinetics:^{25,26}

$$Q_t = kt^{1/2} \quad (1)$$

$$Q_t = kt^n \quad (2)$$

where Q_t is the cumulative percentage of drug release, k is the kinetic constant, n is the release exponent which defines the mechanism of drug release and t is the release time. These equations are generally valid for the first 60% of the total amount of drug release.

2.8 Cell culture test

MC3T3-E1 and RAW 264.7 cells (both obtained from ATCC Cell Biology Collection) were cultured in a modification of Eagle's minimum essential medium (Gibco, Invitrogen, USA), with a volume fraction of 10% fetal bovine serum (Gibco, Invitrogen, USA) under a humidified atmosphere of 5% CO₂ at 37 °C. The medium was refreshed every two days.

2.8.1 Cytotoxicity and ALP activity of daidzein. The cytotoxicity of daidzein was evaluated by means of MTT assay using MC3T3-E1 and RAW 264.7 cell lines. 2000 cells per well in 100 µl culture medium were seeded into 96-well plates and cultured for 24 hours, and then the supernatant was replaced with fresh medium (99.5 µl culture medium + 0.5 µl DMSO containing different concentrations of daidzein). The detailed composition of fresh medium is listed in Table S2 (ESI†) with daidzein concentrations of 25, 5, 1, 0.2, 0.04, 0.008 and 0 µg ml⁻¹. The addition of DMSO was used to avoid the adherence of hydrophobic daidzein on the well plate, and it was considered that the concentration of DMSO used in this study (0.5 vol%) was negligible to induce a toxic effect, as predetermined by the MTT test in Fig. S1 (ESI†). After further 48 h of culture, 20 µl MTT (5 mg ml (ESI†) in PBS) was added to each well and incubated for another 4 hours. Then the supernatant was removed, and 100 µl of lysis buffer (99.4 ml DMSO, 0.6 ml HCl and 10 g SDS) was added per each well with thorough pipetting to dissolve the formazan crystals. Absorbance was measured at 570 nm using an ELISA reader (Analytik Jena AG, Germany). The final readings were the averages of six samples for each daidzein concentration. Alkaline phosphatase (ALP) activity of MC3T3-E1 cells as a function of daidzein concentration was evaluated using an ALP assay kit (Beyondtime Bio-Tech, China). After 96 h of culture with different concentrations of daidzein, the cells were rinsed with PBS and lysed with 0.1% Trion X-100 solution for 30 min at 4 °C. The solution was collected and centrifuged at 3000 rpm for 2 min at 4 °C. 50 µl of the prepared supernatant of each sample was mixed with a 50 µl chromogenic substrate (*para*-nitrophenylphosphate) and cultured for 10 min at 37 °C. Following the incubation, the reaction was stopped by adding 100 µl terminated liquid. The absorbance was measured at 405 nm.

2.8.2 ALP activity and cell morphology on daidzein-loaded coatings. ALP activity and the morphology of MC3T3-E1 cells on multilayer coatings with and without daidzein loading

were studied. Samples were immersed in α-MEM for 2 h in 24-well plates, and then 2×10^4 MC3T3-E1 cells were seeded into each well and cultured for 1 and 4 days. After cultivation, the ALP activity was tested as described previously. For the observation of the cell morphology, the samples were washed with PBS solution, fixed in 2.5 vol% glutaraldehyde for 3 h at 4 °C, rinsed with PBS twice and dried at room temperature for 24 h. After being completely dried, the morphology of the cells was observed by SEM.

3. Results and discussion

3.1 Optimization of the microsphere fabrication process

The processing parameters which were considered to optimize the microsphere fabrication process are listed in Table S1 (ESI†). The obtained samples were observed using an optical microscope, and the particle size distribution was given as mean ± standard deviation, as shown in Fig. S2 (ESI†). It was observed that the particle size was gradually reduced with an increasing stirring rate from 3500 rpm (Fig. S2(a–c), ESI†) to 11 000 rpm (Fig. S2(g–i), ESI†). Meanwhile, a wider particle size distribution was observed when increasing PHBV concentration to 3% w/v and 5% w/v, as shown in Fig. S2(b) and (c) (ESI†), which is likely due to the higher viscosity obtained with higher polymer concentration.⁷ Lower polymer concentration is beneficial for obtaining microspheres with narrower particle size distribution, which however will lead to a lower yield. Therefore, 3% w/v PHBV was considered a compromise leading to a suitable particle size and yield. It has been reported that an insufficient surfactant concentration will lead to a wide particle size distribution and even an irregular morphology of the microspheres,⁷ while higher surfactant concentrations will inevitably introduce more residual surfactant on the surface of the microspheres. It was observed that an increased PVA concentration, from 1% w/v to 3% w/v, led to a more homogeneous particle size distribution. However, there was no significant difference between the size distributions of microspheres prepared with 2% w/v (Fig. S2(j), ESI†) and 3% w/v PVA (Fig. S2(e), ESI†), which indicates that 2% w/v of PVA is sufficient to stabilize the emulsion process. In summary, the parameter combination of 11 000 rpm, 3% w/v PHBV and 2% w/v PVA concentrations was chosen to prepare daidzein loaded PHBV microspheres for the following studies.

3.2 Morphology and size distribution of PHBV microspheres

The typical morphology and size distribution of PHBV microspheres prepared with 5 wt% and 10 wt% of daidzein were observed by SEM, as shown in Fig. 3. All microspheres presented a quite uniform surface morphology and size distribution. The particle size of microspheres prepared with 5 wt% and 10 wt% daidzein is 1.7 ± 0.9 µm and 1.9 ± 1.0 µm, respectively. The width of the particle size distribution, i.e., *span* ($(D_{90} - D_{10})/D_{50}$, where D_i means i % of microspheres lies below the size D_i) is 1.4 for microspheres prepared both with 5 wt% and 10 wt% daidzein. It is well-known that the morphology and size distribution of microspheres have a significant influence on their

Fig. 3 SEM images of PHBV microspheres prepared with (a) 5% and (b) 10% of daidzein. The insets show the particle size distribution of the prepared microspheres.

drug release profiles.²⁷ The relative rough surface topography of daidzein-loaded PHBV microspheres in Fig. 3 could be further optimized to achieve a comparable surface morphology to daidzein-free microspheres. The encapsulation efficiency of daidzein was 71% and 54% when 5 wt% or 10 wt% daidzein was added to PHBV solution, respectively, and the corresponding actual loading of daidzein was in each case 3.38 wt% and 4.91 wt%. It is interesting to note that Ma *et al.*²⁸ reported that the encapsulation efficiency of daidzein-loaded phospholipid complex PLGA nanoparticles and daidzein-loaded cyclodextrin inclusion complex PLGA nanoparticles, prepared by emulsion-solvent evaporation, could reach as high as ~83%, however the encapsulation efficiency of daidzein-loaded PLGA (only) nanoparticles was <25%.

3.3 EPD of alginate–PVA–PHBV microsphere composite coatings

SEM images of the deposited composite coatings with different microsphere concentrations are shown in Fig. 4. The microspheres are seen to be homogeneously distributed in the alginate matrix. Some microspheres were totally embedded in the matrix while others were only partially coated, leading to rough surfaces of the obtained coatings. The relative content of microspheres in the coating was significantly increased when applying higher concentration of microspheres in suspension, and the number of agglomerations was seen to slightly increase at the same time. All composite coatings appeared to be dense and compact, and no microstructural defects such as large pores or micro-cracks were observed. The homogeneous distribution of drug-loaded microspheres provided a uniform release of drug molecules from different areas of the coating. The presence of firmly embedded microspheres in the polymer matrix confirmed also the co-deposition mechanism. In this way, drug molecules pre-loaded in the microsphere have to diffuse firstly through the PHBV matrix and then through the alginate shell to reach the external medium. Therefore, it was expected that a controlled drug release behavior could be obtained from the proposed composite drug delivery system.

The surface topography of the electrophoretic coating, influenced by the addition of microspheres, was evaluated by means of confocal microscopy. As shown in Fig. 4, the 3D topography of each coating is indicated in the form of height distribution,

and the arithmetic mean value (R_a) and normal distributions (gray pattern next to the scale bar) of roughness are also shown. With the increase of microsphere concentration, the samples presented an increasing amount of needle-like tips and a broader roughness distribution, which are mainly due to the presence of microsphere projections on the coating surface. It has been reported that an increased surface roughness can effectively promote initial osteoblastic cell adhesion and proliferation directly *via* enhanced formation of focal contacts or indirectly through selective adsorption of serum proteins relevant for cell attachment.²⁹ Therefore, it can be concluded that the simple control of the surface topography by varying the microsphere/polymer ratio as considered in this study offers a straightforward approach to improve the osteoconductivity of this class of drug delivery coating, especially for antibacterial coatings which may exhibit a certain extent of cytotoxic effect *in vivo*.³⁰

3.4 Multilayered composite coatings by LbL deposition

Surface and cross-sectional SEM images of the deposited coatings at different coating stages are shown in Fig. S3 (ESI†). It was observed that microspheres were completely embedded in the polymer matrix. Moreover, the surface roughness, which was also quantitatively determined in the form of R_a values as indicated in the corresponding figures, was significantly reduced from $1.07 \pm 0.02 \mu\text{m}$ for the EPD coating to $0.63 \pm 0.04 \mu\text{m}$ after the LbL deposition. In this case, the embedded microspheres could be more tightly covered. As shown in Fig. S3 (ESI†), the cross-sections are rather uniform in thickness. Increased thickness of the deposits was observed with additional polymer layers. The coating exhibited a compact and crack-free microstructure with a few microspheres interspersed inside.

3.5 Degradation study

Fig. 5 shows the weight loss of the final multilayered coating as a function of incubation time in PBS for 1, 4, 7, and 14 days in order to quantitatively evaluate their degradation rate. Significant weight losses were observed after incubation for only 1 day, which is mainly attributed to the rapid water-swelling and dissolution of the superficial chitosan–alginate layer under orbital shaking conditions, and subsequent detachment of a few microspheres. However, the degradation rate became slower and stable after the 1st day of incubation, *e.g.* 18 wt% of the total weight loss

Fig. 4 SEM images and 3D topography by confocal microscopy of electrophoretic coatings with different microsphere concentrations (g l^{-1}).

Fig. 5 Weight loss and water contact angles of the multilayered coatings as a function of degradation time in PBS.

was detected during the degradation period from 1 to 14 days, which is highly beneficial for a sustained release of the incorporated drug. According to the water contact angle measurement,

also indicated in Fig. 5, all samples presented hydrophilic contact angles, probably due to the dominant content of hydrophilic polymers (alginate and PVA). The contact angle of the multilayered coating was measured to be $62 \pm 2^\circ$ (not shown in the figure), and the contact angles of pure PHBV, chitosan and alginate films prepared by solvent casting were $100 \pm 4^\circ$,⁹ $103 \pm 5^\circ$ ¹⁸ and $28.3 \pm 3^\circ$,¹² respectively. It was observed that the contact angle of the coating decreased gradually with time of immersion in PBS indicating a more hydrophilic surface during the degradation. The contact angle of a surface is related to several characteristics of the surface including surface composition, roughness and porosity.^{12,21,31} Considering the relatively slow degradation rate of the incubated sample, the reduced water contact angle could be ascribed to the modification of the coating components, *e.g.* the relative amount of hydrophobic components including chitosan and PHBV microspheres decreased during degradation, and the coating became more hydrophilic due to the dominant effect of the alginate phase.

Fig. 6 Surface and cross-sectional SEM images of the deposited coatings after (a and b) 1, (c and d) 7, and (e and f) 14 days of degradation in PBS, the arrows in (b, d and f) indicate the thickness of the degraded coatings.

Both the surface and cross-section of the prepared multilayer coatings were evaluated by SEM (Fig. 6) in order to investigate the evolution of microstructures as a function of degradation time in PBS. An increase of surface roughness, in comparison with the surface of the as-prepared coating, as shown in Fig. S3(c) (ESI[†]), was seen to occur after 1 day of degradation. A relatively high amount of microspheres was exposed on the surface after 1 day of PBS incubation due to the rapid dissolution of the upper coating layers. This finding is also in agreement with the fact that a significant weight loss was detected after 1 day of immersion in PBS. The longer degradation time led to a slight reduction of the surface roughness from $0.97 \pm 0.04 \mu\text{m}$ to $0.85 \pm 0.03 \mu\text{m}$. All samples presented a similar surface morphology, which is considered the result of a slow degradation rate. It should be noted that the cross-sections were prepared by direct mechanical cutting (by scissors) of the coated substrate, nevertheless, the coating was only slightly detached from the substrate indicating, qualitatively, a strong adhesion of the degraded coating to the substrate.

3.6 Adhesion strength

The adhesion strength of the deposited coatings at different coating stages was quantitatively evaluated by means of a scratch tester. The cross on the fracture line, standing for the position of the first detachment under a linearly increased load, was used to determine the critical load in this study. Fig. 7(a–f) shows the fracture lines of the prepared coatings with different additions of PHBV microspheres, number of layers and degradation time. The different fracture morphologies in the figure are probably due to their different failure modes under the linear shear force. Wong *et al.*³² investigated the possible surface

Fig. 7 Typical optical images (a–f) and the corresponding critical loads (g) of the scratch line on the coatings at different processing or degradation stages, (a) A-0.5 g L⁻¹, (b) A-1 g L⁻¹, (c) A-2 g L⁻¹, (d) B, (e) C, (f) C-7d. The crosses show the occurrence of the first detachment applied to determine the critical load.

damage mechanisms that occur during the mar-scratch transition of polymer coatings. A mar is a mark caused by a sliding body that is too shallow to be perceived by human eyes alone but nevertheless does become visible when present in large quantities (arrowed in Fig. 7(a)). A scratch is a mark that forms visible grooves and/or surface damage, which is the typical damage mode for surfaces that suffer heavy moving loads by swivels or ball bearings (arrowed in Fig. 7(d)).³² The damage modes for the PHBV microsphere-filled polymer matrix (homogeneous alginate–PVA mixture) with different microsphere additions were observed to be distinctly different. A lower addition of microsphere fillers to a homogeneous polymer matrix exhibited a plastic drawing mode, which is evidenced by the higher amount of sliding mars around the fracture line. In contrast, a polymer matrix with higher loading of microsphere fillers showed brittle damage mode. This finding suggests that the addition of PHBV microspheres will alter the mode of scratch damage. Furthermore,

the results indicate that the additional chitosan/alginate layer by LbL deposition induced brittle damage mode. The scratch damage mode of sample C-7d (shown in Fig. 7(f)) is probably due to a PHBV microsphere dominated phase (round dots in the figure) which significantly enhanced the brittleness of the degraded coating.

Fig. 7(g) shows the corresponding critical load of the deposited coating at different processing or degradation stages. An appreciable increase of the critical load is observed with the increased concentration of PHBV microspheres. A further increase of the critical load was obtained after introducing chitosan and alginate layers. The critical load of the multilayer coating exhibited a substantial reduction after 1 day of incubation, which is in agreement with the rapid water-swelling and dissolution of the superficial chitosan–alginate layer. There is no significant difference in critical load among all the incubated samples compared to the original one, suggesting that the adhesion strength is successfully maintained during the degradation period. Therefore, it is considered that the polymer phase, *e.g.* the alginate–PVA matrix in this study, can act as both a covering mask retarding the drug release rate and a binder strengthening the bonding between the drug-loaded microspheres and the substrate. Considering the extreme smooth surface of the 316L SS substrates used in the current study, it is expected that the adhesion strength of the proposed drug delivery coating could be further enhanced by increasing the substrate roughness for better mechanical interlocking at the interface between the coating and the substrate.

3.7 Daidzein release profiles

The drug release profiles of PHBV microspheres prepared with 5 and 10 wt% daidzein are plotted in Fig. 8(a). For microspheres prepared with 10 wt% daidzein, the molecule was released in a controlled manner over a period of over 336 hours (95.2 wt% released at 14 days), while the microspheres prepared with 5 wt% daidzein exhibited a complete release within 120 hours (97.8 wt% released at 5 days). Furthermore, a lowered burst release was detected from microspheres with higher daidzein loading. The initial burst release values (at 1 h) of free microspheres prepared with 5 and 10 wt% daidzein were 12.3% and 10.0%, respectively. After 1 d of incubation, 68.1 wt% of

drug was released from microspheres prepared with 5 wt% of daidzein, compared to 45 wt% of drug released from microspheres prepared with 10 wt% of daidzein. A similar phenomenon has also been reported in previous studies,^{33,34} and a possible reason is that lower drug loading leads to relatively more drug being located in the region close to the surface of the microspheres.³³ The drug release mechanism from microspheres prepared by emulsification solvent evaporation involves two main processes: (i) an initial burst release of drug located close to microsphere surfaces, (ii) a relatively constant drug release stage depending on drug diffusion and microsphere degradation. The processing parameters are considered the key to determine drug release profiles by varying the morphology and size distribution of microspheres as well as the degree of encapsulation of drug molecules. Furthermore, the hydrophilicity of drug molecules plays an important role in the subsequent drug release rate, *e.g.* P(3HB) microspheres loaded with tetracycline or gentamycin (hydrophilic drug) have shown faster release rates than the obtained results in the present study.^{8,35} The relatively higher burst release of those antibiotics-loaded microspheres is because the loaded drugs are likely to be adsorbed on the surface or entrapped in the shallow surface of microspheres. However, due to the hydrophobic character of daidzein and PHBV, only a small amount of the encapsulated daidzein will agglomerate toward the microsphere surface during the intense solvent evaporation.²⁷ It is found from the drug release profile that the initial drug burst release is relatively low ($\sim 10\%$ after 1 h) and a subsequent sustained drug release (over 336 hours) was obtained. It can be concluded that daidzein molecules were mainly entrapped inside the PHBV microsphere matrix leading to sustained release. PHBV microspheres prepared with 10 wt% of daidzein addition were selected for the following EPD process due to their reduced burst release, longer release time and the ability to deliver a higher amount of drug. Fig. S4 (ESI†) shows the fitting of the measured daidzein release from free PHBV microspheres using Higuchi and Peppas equations. For PHBV microspheres prepared with 5% daidzein, a linearity (R^2) as high as 0.98 was obtained using both Higuchi and Peppas equations, and the linearity obtained using Higuchi equation was slightly higher than that of Peppas equation, suggesting that the daidzein release was mainly controlled by diffusion. For PHBV microspheres prepared with 10 wt% daidzein,

Fig. 8 Time-dependent daidzein release profiles in PBS–ethanol medium (75 : 25 vol%) from (a) free microspheres prepared with 5 and 10 wt% daidzein loadings, respectively, (b) microsphere-free (control) and microsphere-containing coatings.

the linearity obtained using Peppas equation was obviously higher than using Higuchi equation. The obtained n value was 0.417, which is close to the characteristic n value for Fickian diffusion (*i.e.*, 0.43 for sphere), indicating a diffusion controlled release mechanism. The components (PBS and ethanol) of the release medium used in this work are non-solvents of PHBV, therefore, the shrinkage or swelling of the polymer is negligible,³⁶ which results in a diffusion controlled drug release behavior, as discussed elsewhere.³⁷

Fig. 8(b) compares the daidzein release profiles from microsphere-free and microsphere-containing coatings, respectively. The concentrations of daidzein in both microsphere-free and microsphere-containing suspensions for EPD are the same at 0.2 mg ml^{-1} , however, the total amount of daidzein released from microsphere-containing coatings is substantially higher than that from microsphere-free coatings. It was observed that the daidzein loaded in microsphere-free coating was completely released in 8 hours, which could be attributed to the degradation of the coating components as well as the rapid diffusion of daidzein molecules in ethanol-containing release medium. A burst release of daidzein was also observed on microsphere-containing coatings during the first 12 hours. Subsequently, a sustained release up to 14 days was observed, indicating that the pre-loading of daidzein in PHBV microspheres is able to suppress the drug release rate. The similar trend of burst release on both samples is probably due to the fact that during the preparation of EPD suspensions, a considerable amount of daidzein diffused out of PHBV microspheres under the magnetic stirring and sonication treatments in a water–ethanol solvent. The leaked daidzein molecules performed the same release behavior as that from the control sample. The unleased daidzein contributed to the sustained release up to 14 days. Further investigation will consider optimizing the preparation process of EPD suspensions to minimize the leakage of daidzein from PHBV microspheres during coating processing.

3.8 Cytotoxicity and ALP activity of daidzein

The viability of MC3T3-E1 and RAW 264.7 cells in contact with different concentration of daidzein was evaluated by the MTT test, as shown in Fig. 9(a) and Fig. S5 (ESI†), respectively. Daidzein exhibited severe cytotoxic effects on both MC3T3-E1 and

RAW 264.7 cells with the concentration higher than $5 \text{ } \mu\text{g ml}^{-1}$, while no significant toxic effects on both cell lines were detected with the concentration less than $1 \text{ } \mu\text{g ml}^{-1}$. Therefore, it should be noted that the daidzein loading in the deposited coating should be carefully adjusted to ensure that a released concentration of daidzein, especially during the burst release stage, will not exceed $1 \text{ } \mu\text{g ml}^{-1}$ to avoid toxic effects. ALP activity is considered a phenotypic marker for the mature osteoblast, which is expected to be up-regulated during the differentiation and early stage biomineralization processes in order to induce the formation of the HA phase.³⁸ The effect of daidzein on osteoblastic differentiation was studied by determining ALP activity of MC3T3-E1 cells incubated with different daidzein concentration, as shown in Fig. 9(b). Culture in the presence of daidzein ($0.008\text{--}0.2 \text{ } \mu\text{g ml}^{-1}$) for 4 days led to a significant increase in ALP activity, while an appreciable reduction of ALP activity was detected when using daidzein concentration higher than $1 \text{ } \mu\text{g ml}^{-1}$. It has been reported that a moderate concentration of daidzein will considerably enhance anabolism of osteoblast cells by elevating the production of bone morphogenetic proteins.³⁹ In addition, Dang *et al.*⁴⁰ reported that daidzein presented a biphasic-dose response to osteogenesis and adipogenesis, that activated osteogenesis (ALP activity and nodule formation) and decreased adipogenesis (the number of adipocytes) occurred when using daidzein concentration below $20 \text{ } \mu\text{M}$ (or $5 \text{ } \mu\text{g ml}^{-1}$), whereas inhibited osteogenesis and stimulated adipogenesis was detected at daidzein concentrations higher than $30 \text{ } \mu\text{M}$ (or $7.5 \text{ } \mu\text{g ml}^{-1}$). The experimental results obtained in the present study show a similar trend to those reported in the literature, and are also indicative of the optimization of the daidzein release profile from the deposited coatings.

ALP activity of MC3T3-E1 cells incubated with daidzein-free or daidzein-loaded multilayer coatings for 1 and 4 days is shown in Fig. 10(a). No obvious increase of ALP activity was observed on the blank well plate after 4 days of incubation. The daidzein-loaded sample shows relatively higher ALP activity than the other two groups after 1 day of incubation. After day 4, significantly higher levels of ALP activity were observed on coating samples as compared to the blank control. In addition, the daidzein-loaded sample presented a higher level of ALP activity than

Fig. 9 The effect of daidzein concentration on (a) cell viability after 2 days of culture measured by MTT assay, and on (b) ALP activity after 4 days of culture (the MC3T3-E1 cell line was used for both tests, error bar represents SD, $n = 6$).

Fig. 10 (a) ALP activity of cells (MC3T3-E1) cultured on multilayer coatings with/without daidzein loading, cell morphologies on (b) daidzein-free and (c and d) daidzein-loaded multilayer coatings after culture for 4 days. The arrows indicate the presence of cells or filopodia.

daidzein-free samples, which indicates that the addition of daidzein leads to enhanced cell differentiation besides the stimulatory effect from the coating itself. Both low and high magnification SEM images of the cell morphology on day 4 samples, cultured with or without loading daidzein, are shown in Fig. 10(b–d). The cells grown on daidzein-free coatings (Fig. 10(b)) exhibit a flattened shape while the cells grown on daidzein-loaded coatings (Fig. 10(c)) presented a more spread morphology and filopodia. It was also observed that the relative cell density on daidzein-loaded coatings is higher than that on daidzein-free coatings, indicating enhanced cell proliferation in the presence of daidzein. Furthermore, it is shown in Fig. 10(d) that cells tightly adhered to the coating surface and tended to cover and engulf the microspheres. Both ALP activity measurements and SEM observations indicate that the proposed drug delivery coating platform, with controlled release of daidzein molecules, has the potential of promoting bone-to-implant integration by accelerating the bone healing process.

4. Conclusions

A multilayered and biodegradable drug delivery coating system consisting of daidzein-loaded PHBV microspheres embedded in a biodegradable polymer matrix was successfully fabricated by a combination of EPD and LbL deposition. The factors affecting the morphology and the microsphere size distribution, including the stirring rate and the addition of PHBV, were optimized to obtain smooth microspheres with a narrow size distribution. A tunable amount of daidzein could be loaded during the microsphere fabrication process, and daidzein was released in a

diffusion controlled manner in the ethanol–PBS mixture medium. In addition, it was found that the free microspheres loaded with 10 wt% of daidzein showed a slower and sustained release up to 14 days compared with microspheres loaded with 5 wt% of daidzein (5 days). SEM observations revealed that microspheres were wrapped by and homogeneously distributed in the polymer matrix. The relative content of microspheres in the deposited coating could be simply adjusted by varying the microsphere concentration in EPD suspensions, which is extremely useful for tailoring accurately the drug loading in the coating. According to the degradation study, all coatings exhibited hydrophilic character with a decreasing contact angle during degradation, and 42 wt% of the coating weight was retained after 14 days of incubation. Moreover, the adhesion strength of the deposited coating to the substrate was maintained during the degradation process. Pre-encapsulation of daidzein in PHBV microspheres showed minimized burst and sustained release compared to microsphere-free coatings. Cytotoxicity tests as a function of daidzein concentration indicated a critical daidzein concentration of $1 \mu\text{g ml}^{-1}$ inducing toxic effects on MC3T3-E1 and RAW 264.7 cells. MC3T3-E1 cells presented significantly higher ALP activity on daidzein-loaded multilayer coatings after 4 days of incubation compared to daidzein-free samples.

Indeed more work is required to further optimize the material structure (both microspheres and coating) and to comprehensively understand the interaction between drug molecules and polymers used. For example, the surface uniformity of free microspheres, which is always associated with an initial burst release effect, should be further optimized to produce smoother microspheres. The degradation rate of the prepared multilayer coating should also be adjusted to avoid severe weight loss during

the initial incubation stage. Summarizing, the drug delivery system proposed in this study provides a straightforward pathway for delivery of a variety of biological molecules for biomedical applications.

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