Polyelectrolyte Coated PLGA Nanoparticles: Templation and Release Behavior

Jie Zhou, Sergio Moya, Lie Ma, Changyou Gao, Jiacong Shen

Poly[(D,L-lactide)-co-glycolide] nanoparticles coated with polyethyleneimine on their surface were prepared by an emulsification-solvent evaporation method and subsequently surface modified by LBL assembly. The assembly of poly(acrylic acid) and polyethyleneimine on a planar substrate and on the PLGA nanoparticles was monitored by QCM-D, ζ-potential, flow cytometry and TEM. Carboxylic and amino groups in the multilayers were crosslinked by carbodiimide condensation, which was also later used to graft poly(ethylene glycol) (PEG). Rhodamine 6G, 5(6)-carboxyfluorescein and fluorescein were incorporated into the nanoparticles and their release profiles were recorded at 60 °C and at 37 °C for rhodamine 6G, for nanoparticles with a multilayer coating, and those that were crosslinked and grafted with PEG.

Introduction

Biodegradable polymeric micro- and nanoparticles, especially those made of polylactide (PLA), polyglycolide (PGA) and their copolymer poly(lactide-co-glycolide) (PLGA), because of their good biocompatibility, low cytotoxicity and easy preparation, are widely used in the therapeutic field as carriers for drug, gene and vaccine delivery. The fabrication and optimization of such carriers is of enormous importance for developing health therapies, and in the last decade has triggered much research devoted to controlling the release rate, targeting and prolonging the circulation time of drug delivery vehicles.

There are several convenient methods that have been used to prepare PLA and PLGA nanoparticles, such as O/W and W/O/W emulsification-solvent evaporation, nanoprecipitation and supercritical fluid technology, among which the O/W and W/O/W emulsification-solvent evaporation methods are the most frequently employed. A very difficult task concerning the templation of PLGA nanoparticles is the engineering of the particles in such a way to achieve control of the release rate of drugs entrapped in the particles. Several strategies for controlling the release of PLA PLGA nano- or microparticles, have been developed, such as varying the size of the particles, triggering release with pH or temperature and preparing mixed particles with the addition of other polymers etc.

The surface modification of PLA or PLGA nanoparticles provides a route to prolong particle circulation time or target specific organs or tissues. A major challenge in the templation of drug delivery systems is the control of their release behavior and at the same time achieving a functional surface, which assures a targeted delivery.

Layer by layer (LBL) assembly has proved to be a powerful technique for surface modifications. The LBL technique involves the alternative assembly of oppositely...
charged polyelectrolytes on planar or curved charged surfaces, resulting in a thin polymer film of a few nanometers thickness. The thickness and composition of the LbL films can be controlled with nanoscale precision. Multi-compartmentation and multifunction can also be endowed by incorporating various substances, such as nanoparticles, lipids, proteins and DNA, into or onto the multilayers, or by replacing selected polyelectrolyte layers. Several examples of LbL applications in the biomedical field should be mentioned, such as loading drugs into multilayer microcapsules and the incorporation of cell growth factors into 3D scaffolds for tissue engineering.

LbL films can also function as barriers to control permeability based on considerations of chemical architecture and charge interaction. Some elegant examples have been documented. For example, Shi and Caruso used pyrene microcrystals as templates to assemble PSS/PAH multilayers. They found that the release profiles and rates of pyrene were controlled by the layer number. De Geest applied three layers of PSS/PAH on the surface of self-rupturing microgels made from dextran-hydroxyethyl methacrylate, and found that the multilayers had a major impact on the release features of the loaded substances. Chemical functionalization and control over the permeability provided by the multilayers should be an extremely useful and easy way to engineer PLGA nanoparticles for targeted and controlled drug delivery. For instance, reactive amine or carboxylic groups can be brought onto the PLGA nanoparticle surface by LbL assembly, which can be used to attach other functional moieties, such as poly(ethylene glycol) (PEG) or cell specific antibodies, without influencing the stability of the PLGA nanoparticles. The density of the functional moieties is easily mediated by adjusting the assembled layer number.

In this work, PLGA nanoparticles were prepared by the W/O emulsification-solvent evaporation method using a polyethyleneimine (PEI) solution as the water phase, to provide the PLGA nanoparticles with amine groups on their surface during particle preparation. Poly(acrylic acid) (PAA)/PEI multilayers were then assembled onto the PLGA nanoparticles into films of varying thickness and were, in some cases, chemically crosslinked.

The particular choice of PAA and PEI was due firstly to the carboxylic and amine groups present in these polymers, which can be easily functionalized, and also that both polymers, especially PEI, show an acceptable degree of biocompatibility. Amino-terminated PEG was also grafted to the multilayers. The assembly process and the multilayer stability on planar substrates and PLGA nanoparticles were characterized by QCM-D, flow cytometry and z-potential. Finally, three different dyes, rhodamine 6G, fluorescein and 5(6)-carboxyfluorescein (Figure 1), were entrapped inside the PLGA nanoparticles to study their release behavior for the different LbL coatings. The release behavior of PLGA nanoparticles with incorporated rhodamine 6G was first studied at 37 °C to investigate the mechanism of release under physiological conditions, and then at 60 °C, a temperature higher than the glass transition temperature ($T_g$) of the PLGA used. The release at 60 °C was faster (hours) than at 37 °C and, although for practical usage a temperature closer to body temperature would be more useful, we found it easier to work in conditions of fast release as we wanted to compare how the different surface architectures of the PLGA nanoparticles affected the release.

### Experimental Part

#### Materials

Poly[(D,L-lactide)-co-glycolide] (PLGA) (D,l-lactide 90: glycolide 10) with an average molecular weight of 100 kDa was purchased from the China Textile Academy. Poly(acrylic acid) (PAA, $M_w$ ≈ 5 kDa) was purchased from Acros, and branched polyethyleneimine (PEI, $M_w$ ≈ 25 kDa), Jeffamine ED-2001 (PEG terminated with amino groups, $H_2N$–PEG–$NH_2$, $M_w$ ≈ 1.9 kDa), phosphate buffer saline (PBS), rhodamine 6G, fluorescein, 5(6)-carboxyfluorescein, ethylcarbodiimide hydrochloride (EDC) and $N$-hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich. All chemicals were used as received.

#### Preparation of PLGA Nanoparticles

PLGA nanoparticles were prepared by the O/W emulsification-solvent evaporation method. Firstly, 1 mL of 20 mg·mL$^{-1}$ PLGA dichloromethane solution (organic phase) was added to 4 mL of 5% PEI solution (water phase), which was then emulsified by an ultrasonicator (SONIC$^\text{Tm}$ VCX 500) for 20 s. This emulsion was immediately poured into 100 mL of distilled water, and stirred for 4 h with a magnetic stirrer until the organic solvent totally evaporated.
evaporated. The PLGA nanoparticles were collected by centrifugation at 10,000 g for 5 min, and were washed with MilliQ water 5 times to remove the free PEI initially presented in the water phase. The samples for dye release experiments were prepared by adding 0.5 mg of dye (rhodamine 6G, fluorescein or 5(6)-carboxyfluorescein) into the organic phase before sonication. About 100 μL of ethanol were added to increase the solubility of fluorescein and 5(6)-carboxyfluorescein in the dichloromethane.

**LbL Assembly on the PLGA Nanoparticles**

1 mg·mL⁻¹ PAA and PEI solutions in 10⁻³ M PBS were employed for the coating. The pH of the solution was adjusted to 7.4 by the addition of 1 M HCl or NaOH. The PLGA nanoparticles were incubated in the polyelectrolyte solution for 15 min, centrifuged, dispersed in PBS, centrifuged and dispersed again. This procedure was repeated 3 times for each layer deposition.

The surface crosslinking and the grafting of PEG was performed in two steps. Firstly, the PLGA nanoparticles coated with 2.5 bilayers of PAA/PEI (PAA as the outermost layer) were incubated in 10⁻³ M EDC and 10⁻³ M NHS solution at pH 6.5 for 30 min to activate the carboxylic groups on the surface. After separation by centrifugation, the particles were redispersed in 10⁻³ M EDC and 10⁻³ M NHS solution at pH 8.6 for crosslinking, or in 5 mg·mL⁻¹ H₂N–PEG–NH₂, 10⁻³ M EDC and 10⁻³ M NHS solution at pH 8.6 for PEG grafting, each for 40 h. After the crosslinking or PEG grafting, the nanoparticles were dispersed in a pH 12 NaOH solution for 5 min to deactivate the carboxylic acid. Finally, the particles were washed 5 times with PBS and recovered by centrifugation.

**Characterization of the Assembling Process**

An E4 Quartz Crystal Microbalance with Dissipation (QCM-D) from Q-Sense, Göteborg, Sweden, was employed to follow the multilayer assembly. Gold coated quartz crystals (5 MHz) were used as substrates. An initial layer of PEI was assembled, on top of which 10⁻³ M EDC and 10⁻³ M NHS solution was added. The fluorescence intensity was recorded after each layer deposition and washed 3 times with PBS. All the z-potentials were measured with a Fluorolog®-3, (HORIBA Jobin Yvon Inc, USA), exciting at 530 nm and measuring at 550 nm. After release, all the PLGA nanoparticles were analyzed by an E4 Quartz Crystal Microbalance with Dissipation (QCM-D) from Q-Sense, Göteborg, Sweden.

**Release Profiles from PLGA Nanoparticles**

Firstly, the release behavior of the PLGA nanoparticles with entrapped rhodamine 6G was studied at 37 °C. About 2 mg of PLGA nanoparticles coated with increasing numbers of polyelectrolyte layers (0, 1, 2, 3, 4, 5, 6, 7) were weighed and suspended in 1 mL of 10⁻³ M PBS, then at designed time intervals, the nanoparticles were centrifuged at 10,000 g for 3 min, and 100 μL of supernatant were taken out and another 100 μL of fresh PBS solution were added. The fluorescence intensity was recorded with a Fluorolog®-3, (HORIBA Jobin Yvon Inc, USA), exciting at 530 nm and measuring at 550 nm. After release, all the PLGA nanoparticles were analyzed by an E4 Quartz Crystal Microbalance with Dissipation (QCM-D) from Q-Sense, Göteborg, Sweden.

**Results and Discussion**

**Characterization of the Uncoated PLGA Nanoparticles**

The surface morphology of the uncoated PLGA nanoparticles was characterized by TEM. A rough and coronal surrounding surface was found for these PLGA nanoparticles (Figure 2A), whose size was around 276 nm (Figure 3). However, the diameter of the nanoparticles measured by DLS (346 ± 12 nm, Figure 3) was much larger. z-potential measurements of the nanoparticles recorded a positive value of ≈+12 mV (Figure 4). UV spectroscopy...
measurements provided a value of PEI of 70 μg·mg⁻¹ for the PLGA nanoparticles, a higher value than previously reported.[30] All these results confirm the successful incorporation of PEI molecules onto the PLGA nanoparticle surface. The larger diameter measured by DLS compared with that measured by TEM is due to the different state of the particles during these measurements. To understand these differences we must take into consideration two things. Firstly, DLS measures the hydrodynamic diameter, i.e., the particle size including the hydrophilic hairy layer of highly hydrated PEI around the particles, whereas TEM measures only the solid part of the particles in the dry state, in which the hairy PEI collapses, increasing density and losing entrapped water. Secondly, it is also likely that the PEI molecules do not form a homogeneous coating on the nanoparticles, with some chains longer than the rest and protruding into the solution.

LBL Assembly

To monitor and confirm the LbL assembly process, the assembly was first conducted on a gold coated quartz crystal in a QCM-D chamber. Figure 5(a) shows a continuous negative variation in frequency (∆f) of −44 Hz during the deposition of the first 4 layers, revealing that the mass was also continuously increasing. Then, a zigzag change in frequency was observed, i.e., a decrease of ∆f of 15 Hz after each deposition of PAA was followed by an increase of ∆f of 12 Hz after adsorption of PEI; the mass changes during the assembly calculated with the Sauerbrey equation are also shown in the inserted graph in Figure 5(a). This result reveals that upon PEI deposition part of the PAA was removed. This process is most likely attributable to loosely bound PAA molecules, a phenomenon observed for other weak polyelectrolyte multilayers.[33] The dissipation curve (Figure 5(b)) confirms this assumption. Dissipation values are much smaller after PEI deposition than that after PAA assembly, suggesting that the multilayers are more condensed after PEI assembly. The ζ-potential (Figure 4), which reflects the charge of the outmost layer, changed reversibly along with the assembly, i.e., 12 and −22 mV when PEI and PAA are the outmost layer, respectively, in PBS. These results confirm that, although partial removal of PAA takes place during the
assembly, the alternating deposition of PAA/PEI layers is continuous.

The assembly on the PLGA nanoparticles was further traced by flow cytometry, employing FITC labeled PEI (FITC-PEI) and non-labeled PAA as multilayer constituents. Flow cytometry allows the changes in fluorescence to record intensity in the PLGA nanoparticles after the deposition of FITC-PEI layers. In Figure 6, the mean fluorescence intensity of a population versus fluorescence distribution from flow cytometry measurements has been plotted as a function of the assembled polyelectrolyte layers on the PLGA nanoparticles. The initial point in Figure 6 corresponds to the uncoated PLGA nanoparticles, where almost no fluorescence could be recorded. Almost no fluorescence could be recorded after the deposition of one PAA layer as well. Measurements were performed after assembling each FITC-PEI layer and also after each PAA layer. The fluorescence intensity increased with the assembly of each PEI layer but also decreased with each PAA layer. The highest fluorescence intensity is indeed found for the first FITC-PEI layer. The subsequent deposition of a PAA layer results in a reduction of the total fluorescence of 4/5 of the previous value. For the multi-layers, with the FITC-PEI as the outer layer, the fluorescence intensity was much stronger than when PAA was the outer layer. This means that when PAA was assembled on the FITC-PEI surface, some of the FITC-PEI was removed from the surface. The change in fluorescence could also be due to quenching caused by a change in the environment of FITC-PEI. Anyway, the total amount of FITC-PEI was increasing, since the intensity kept increasing for the samples with odd numbers of layers (the dashed line in Figure 6).

After assembly of the PAA/PEI multilayers, no significant alteration of the particle size was seen by either TEM or DLS (Figure 2 and 3), but the particle surface looked smoother. The PEI content (Figure 7) measured by UV-vis spectroscopy was found to be about 70 μg·mg⁻¹ of PLGA.
nanoparticles. Further assembly of the multilayers did not improve the PEI content, but instead reduced the amount slightly. This is reasonable since the original PEI amount is large, corresponding to a thickness of about a hundred nanometers when hydrated. The slight decrease after assembly should be a result of removal of the loosely attached PEI on the PLGA nanoparticles.

Therefore, the growth mechanism of the multilayers can be determined (Figure 8). Firstly, after deposition of PAA or PEI, a partial removal of the previous layer occurs, indicating that a loosely bound polyelectrolyte layer always exists regardless of the charge of the layer. This loosely bounded layer is partially removed upon deposition of the next layer. The reason is probably that, since both PAA and PEI are weak polyelectrolytes, and at pH 7.4 cannot be fully charged, it is a weak combination between them. The high value of fluorescence for the first deposited PEI layer cannot be correlated with the QCM-D experiments. We have attributed this to the PEI used as a stabilizer for the PLGA nanoparticles, which forms a sort of brush around the nanoparticles. Consequently, the first PAA layer and presumably the next PEI layer can be largely adsorbed in between the chains of the brush.

PEG grafting is one of the most efficient ways to prolong the circulation time of delivery systems because of its low protein affinity. In this work, amino terminated PEG was grafted onto the multilayer (PAA as the outmost layer) coated PLGA nanoparticles by means of EDC/NHS condensation. The changes in ζ-potential in Figure 9 illustrate the successful grafting. The ζ-potential of the PLGA nanoparticles coated with (PAA/PEI)2/PAA multilayers measured in 10 × 10−3 M NaCl at pH 7.4 (around −40 mV) was much lower than that measured in PBS (−22 mV). After crosslinking, the ζ-potential increased to −18 mV. Two interpretations can be given to explain this change in the potential. It may be that some of the outer carboxylic groups have been crosslinked, with a subsequent change in the PEI and PAA layers.
in density of functional groups. It could also be that, after the crosslinking, the depth of flow penetration was thinner, reducing the effective contribution of more deeply located carboxylic groups to the $\zeta$-potential.[37] Then, after PEG grafting, the change in functional group density or the hydrodynamic screening[37] brought by PEG or both of them were more effective, so $\zeta$-potential increased even more than the crosslinked nanoparticles, going down to around $-12$ mV.

**Release Profiles**

The release behavior of rhodamine 6G from PLGA nanoparticles coated with increasing number of polyelectrolyte layers was characterized in PBS at 37 °C. The release curves are shown in Figure 10(a). From these curves, a release rate of almost zero order release can be observed, with just a little burst release, less than 5% of the amount of dye, at the very beginning. The release curves can be divided into two groups: those of nanoparticles with PEI as the outermost layer (non-LbL coated nanoparticles are also included), over the gray dash line, and, on the other hand, the release curves of nanoparticles with an outermost layer of PAA, under the gray dash line. The rate of release (Figure 10(b)) can be obtained after fitting the release curves with a zero order release model. The difference in release rates between samples with PAA and PEI as the outermost layer is remarkable. The release rates were much lower when PAA was the outmost layer ($\approx 0.009–0.01$ d$^{-1}$), approximately $\approx 50–65\%$ of the values for PEI ($\approx 0.005–0.0067$ d$^{-1}$). If one compares samples with different layer numbers but the same outermost layer, it can be deduced that the release rate decreases with increasing layer number; this effect is, however, more pronounced for samples with PAA as the outermost layer than for those with PEI. The slow rate of release of rhodamine 6G from PLGA NPs at 37 °C is probably due to the hydrophobic character of the rhodamine 6G and to the fact that the PLGA is at a temperature below the glass transition, resulting in very little mobility of the polymer molecules in the matrix. The differences in the release observed for PEI and PAA results from the complication of PAA with the positively charged rhodamine 6G, which must diminish diffusion from the nanoparticles.

Considering possible applications of these nanoparticles for the temperature trigged release of anti-tumor drugs release, studies were performed at high temperature (60 °C) for three dyes displaying different degrees of charge: rhodamine 6G (positive charge in PBS); fluorescein (almost neutral in PBS); 5(6)-carboxyfluorescein (negative in PBS). These were carried out in a 60 °C water bath. The profiles are shown in Figure 11. Each of the three dyes showed a different release profile. Firstly, the total released amount of dye was different in each case, as it was the loaded amount of dye. While more than 90% of fluorescein (loading ratio of 0.01 wt.-%) was released from the PLGA nanoparticles after 24 h, less than 60% of rhodamine 6G (loading ratio of 0.08 wt.-%) and only 35% of 5(6)-carboxyfluorescein (loading ratio of 0.13 wt.-%) were released during the same time. These results indicate that the rhodamine 6G and 5(6)-carboxyfluorescein, even with a higher loading ratio, have a stronger interaction with the PLGA matrix or the multilayer coated PLGA nanoparticles than that of the fluorescein. The difference in loading makes it difficult to compare the release of one dye to another, bringing into consideration an extra parameter. Nevertheless, the intention here is to compare how the different coatings of the nanoparticles play a role in the release for three different kinds of dyes, one non charged, one positively charged and one negatively charged, rather than to make a comparison.

In Figure 11, two types of release profiles can be seen. The release of rhodamine 6G and fluorescein was very fast at the beginning, and then became substantially slower.
after 2 h. Consequently, most of the dyes were released within the first 2 h. Although the release profiles resemble a first order release model or Higuchi model, the data cannot be fitted very well by any of them (data not shown), probably due to the serious burst release. In contrast, the release of 5(6)-carboxyfluorescein was much slower, and can be fitted very well with a zero order release model, except for the initial part, which should be the result of burst release. Here, we chose a temperature higher than that of the glass transition temperature \( T_g \) of the PLGA (55–58 °C). Therefore, in contrast to the glassy state in which the polymer chains are hardly mobile, the segments of the PLGA chains have bigger freedom to move, leading to easier diffusion of dye molecules in the polymer matrix\(^{[3,38]} \) with which it is easier to understand why the release rate was so slow at 37 °C (less than 35% after 30 d release) compared with the same particles at high temperature (at least 40% after 8 h release) from Figure 10(a) and 11. Consequently, the release rates are very fast at the beginning. The rather slow release of 5(6)-carboxyfluorescein can be explained by the charge interaction between the dyes and the PEI hairy layers (7% of the total mass of the PLGA nanoparticles). Due to its positive charge, the PEI layer may form a stronger complex with the negatively charged 5(6)-carboxyfluorescein, which in turn slows down the dye release. However, no interaction or even repulsion exists between the PEI and the rhodamine 6G and fluorescein dyes, leading to faster release.

Figure 12 presents the amount of released dye as a function of layer number. For fluorescein, the released amounts were almost independent of the layer number, implying that the neutral dye does not interact with the polyelectrolytes in the multilayers. According to this result, one can further deduce that the pores present in the multilayers are big enough to permit the free diffusion of fluorescein. The release amounts of 5(6)-carboxyfluorescein and rhodamine 6G, however, decreased slightly and significantly with the increase of layer number, respectively. Interestingly, a lower and higher released amount was recorded when PAA and PEI were the outmost layers, respectively. It is assumed that in the polyelectrolyte multilayers most of the charges are compensated, whereas the outermost layer decides the charging state of the whole multilayer.\(^{[39]} \) Consequently, the positively charged rhodamine 6G binds to the multilayers to a larger extent when PAA is the last layer. This binding may then more or less retard the further release of the internal dyes from the colloids. This phenomenon is not observed for 5(6)-carboxyfluorescein. A possible reason could be that the
high amount of PEI in the nanoparticles makes the effect of PAA very small. Experiments with other polyelectrolyte pairs are underway to better understand this behavior.

Crosslinking of the multilayers slowed down the release of the dyes because the multilayer structure becomes denser. This effect is not obvious for rhodamine 6G, but is prominent for 5(6)-carboxyfluorescein and especially for fluorescein (20% reduction). Since the release amount of rhodamine 6G is already small when PAA is the last layer, the crosslinking does not further affect release.

**Conclusion**

In this work, we have shown that PEI can be used as a stabilizer to prepare PLGA nanoparticles using the O/W emulsification-solvent evaporation method. The PEI decorated PLGA nanoparticles were then modified with PAA/PEI multilayers using the LbL assembly technique, whose structure can be further modified with PEG and EDC/NHS crosslinking. The structure of the assembled multilayers was characterized by QCM-D, flow cytometry, z-potential measurements and absorbance spectroscopy, showing that removal of part of the previously adsorbed layer takes place upon deposition of a new layer. These results reflect the weak binding between the PAA and PEI pairs. Fluorescence dyes with different kind of charges, i.e., rhodamine 6G, fluorescein and 5(6)-carboxyfluorescein, were incorporated into the PLGA nanoparticles. Release experiments at 60 and at 37°C were performed. At the lower temperature, the release curves showed that the release behavior of all the PLGA nanoparticles with different layer number multilayers could be fitted very well with a zero order release curve, and that the release rate was much lower than for PAA as the outmost layer of the particles, with the more layers, the lower the release rate. At high temperature, release experiments showed that all the dyes can be released. While a very prominent burst release was observed for rhodamine 6G and fluorescein, this was not present for 5(6)-carboxyfluorescein. For most of the dyes, crosslinking of the multilayers decreased their released amounts, in particular for the fluorescein. These results are helpful in understanding the nature of the weak polyelectrolyte PAA/PEI multilayers, and help to use multilayers to control the release profile of polymeric nanoparticles.

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