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# Layer by layer surface engineering of poly (lactide-*co*-glycolide) nanoparticles: A versatile tool for nanoparticle engineering for targeted drug delivery†

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Recent work regarding the Layer by Layer (LbL) engineering of poly(lactide-*co*-glycolide) nanoparticles (PLGA NPs) is reviewed here. The LbL engineering of PLGA NPs is applied as a means of generating advanced drug delivery devices with tailored recognition, protection, cargo and release properties. LbL in combination with covalent chemistry is used to attach PEG and folic acid to control cell uptake and direct it towards cancer cells. LbL coatings composed of chitosan and alginate show low protein interactions and can be used as an alternative to Pegylation. The assembly on top of LbL coatings of lipid layers composed of variable percentages of 1,2-dioleoyl-sn-glycero-3-choline (DOPC) and 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS) increases NP uptake and directs the NPs towards the endoplasmic reticulum. The antibody anti-TNF-α is encapsulated forming a complex with alginate that is assembled LbL on top of PLGA NPs. The antibody is released in cell culture following first order kinetics. The release kinetics of encapsulated molecules inside PLGA NPs are studied when the PLGA NPs are coated via LbL with different polyelectrolytes. The intracellular release of encapsulated Doxorubicin is studied in the HepG2 cell line by means of Fluorescence Lifetime Imaging.

**layer by layer, PLGA NPs, cell uptake, antibody delivery, lipid layers, intracellular release**

## 1 Introduction

Polymeric nanoparticles (NPs) are appealing systems for drug delivery since the size, amount of loaded material and release features can be well controlled [1–10]. Among polymeric NPs, the homopolymer of lactic acid (PLA) and its copolymer, poly(lactide-*co*-glycolide) (PLGA), have gained significant attention from the scientific community in recent years [10–18]. PLA and PLGA NPs are easy to produce and display excellent biocompatibility and biodegradability. PLA and PLGA NPs are prepared following different routes such as simple or double emulsion-solvent evaporation, nanoprecipitation, spray drying, etc [19–22]. Both hydrophobic and hydrophilic drugs have been encapsulated inside PLGA NPs [23–26].

Recently, in our group we have explored the use of the so called layer by layer (LbL) technique of polyelectrolyte assembly for the surface engineering of PLGA NPs. LbL is based on the alternate assembly of oppositely charged polyelectrolytes by electrostatic interactions, entropically triggered by the release of counterions. LbL is already a well established and simple technology for the non covalent engineering of surfaces [27–30]. One interesting application of LbL is in the fabrication of polyelectrolyte capsules for drug delivery [31]. Capsules are fabricated after assembling a thin polyelectrolyte film by LbL on top of degradable

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colloids. The supporting colloids are destroyed after film assembly resulting in a free standing closed thin polymer film, a capsule [31, 32]. In recent years, a large body of work has been devoted to the development of these systems and their application in drug delivery [33, 34]. Comparatively less work has been accomplished, however, in the use of the LbL technique for the engineering of colloidal particles for their application in drug delivery. The use of LbL for the surface engineering and modification of PLGA NPs or any other organic NPs has the advantage that it does not affect the stabilisation of the NPs as covalent chemistry may do, and in principle for the application of LbL it is only necessary that the NP surface be charged. LbL coating can be used as a starting point for further modifications as we will show. The functional groups present in the polyelectrolyte chains, often carboxylates or primary amines, can be employed for the covalent attachment of other molecules, and the polyelectrolyte multilayer (PEM) can act as a cushion for the assembly of lipid layers, which in turn can be used for the assembly of other biomolecules. The LbL coating, with subsequent chemical functionalisation, can be used as a means for targeting NPs, for example, enabling selective interactions with cells or guiding them through specific uptake pathways that finally lead the NP to different intracellular regions. Regardless, LbL can also provide a means to protect the NP and its cargo. We have shown that with the use of NPs with LbL coatings based on charged carbohydrates, we have achieved an antifouling effect that also impacts on their cellular uptake. LbL coatings can also be used to control the release of encapsulated molecules depending on their charges. Additionally, the LbL films themselves can encapsulate large therapeutics such as antibodies, RNA or plasmids, carried between layers. Moreover, the LbL coatings on NPs can simultaneously provide protection, targeting and a means for cargo encapsulation.

The main scope of this feature article will be to discuss the benefits and potential applications of LbL films for the surface engineering of PLGA NPs for the fabrication of delivery devices with multiple functions and summarise the work undertaken in this regard within our group. More specifically, we will show LbL coating with chitosan and alginate as an alternative to pegylation, the use of LbL in combination with covalent chemistry, the encapsulation of antibodies and plasmids between polyelectrolytes on top PLGA NPs, and the use of lipid layers on top of the LbL coating for the control of their intracellular targeting towards the endoplasmic reticulum. We will also address the release of encapsulated materials intracellularly that are important for the application of NPs for drug delivery.

## 2 Layer by layer coating of PLGA NPs

In previous studies [35–37] we have shown that the LbL assembly on top of PLGA NPs synthesised by emulsion techniques with a polyelectrolyte i.e. polyethylenimine (PEI) or a protein, Bovine Serum Albumin (BSA), as a stabiliser could be employed as a starting point for the functionalisation of the NPs. LbL coatings of poly allyl amine (PAH) and poly acrylic acid (PAA) provide functional groups, amines and carboxylates, that can be employed for covalent chemistry. In this way, by simple condensation reactions it was possible to attach PEG and folic acid to the NP. The strategy followed is summarised in Figure 1. The polyelec-

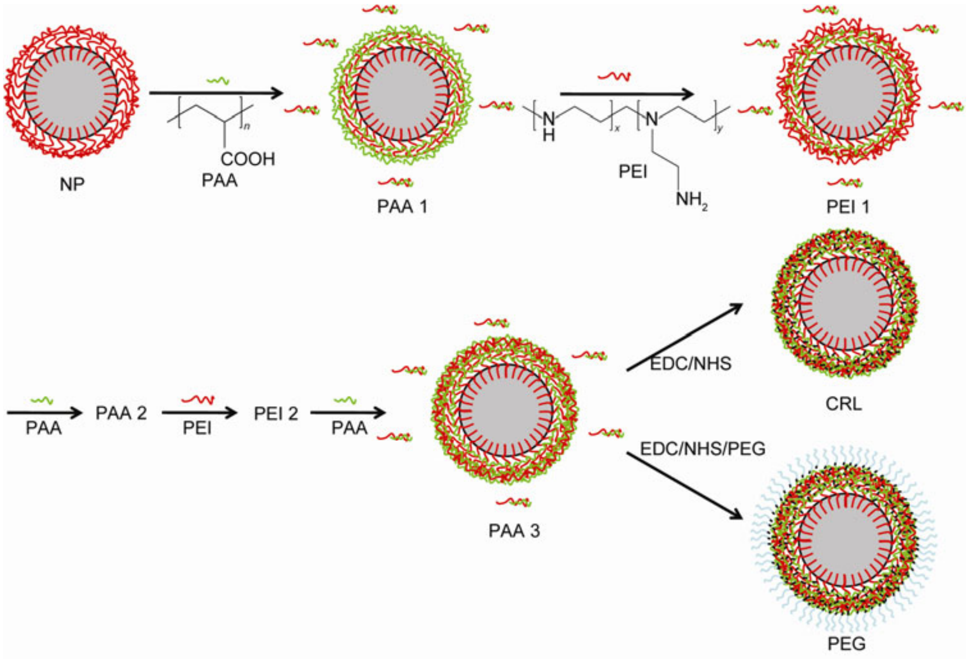

**Figure 1** Scheme of the surface engineering of PLGA NPs with a LbL film of PAA and PEI. Reproduced from Ref. [36].

trolyte layer is used as a functionalisation tool and intermediate step that allows the attachment of molecules to the NPs independently of the actual surface chemistry of the NP.

The attachment of folic acid (FA) that was linked to the PEG helps to increase uptake in cancerous cell lines, which as a rule overexpress folate receptors. The strategy developed was to decrease unspecific interactions due to the charges present in the polyelectrolytes by attaching PEG and then increase specific interaction by adding folic acid. As a proof of principle, the uptake of NPs was studied by Confocal Laser Scanning Microscopy (CLSM) and Flow Cytometry (FACS) as shown in Figure 2.

## 3 LbL chitosan and alginate as an alternative to PEGylation

LbL films have properties which are a combination of the properties of the individual polyelectrolyte components. The use of natural polyelectrolytes, like chitosan and alginate, for the coating of the NPs showed a similar effect to PEGylation, i.e. reducing the specific interaction of the NPs with proteins and cells [37]. Cellular uptake experiments performed both by confocal microscopy and FACS show that the coating of the NPs reduces cellular uptake when compared to the uncoated NPs. Upon the attachment of FA or PEG/FA to the alginate, uptake again increases. The alginate and chitosan coating provides an alternative to the PEGylation, removing one step in the functionalisation process in order to diminish unspecific interactions and to target NPs.

The low interaction with proteins of the LbL alginate/chitosan coatings does not depend on the nature of the outer layer for films with less than 10 layers. This is a surprising result to a certain extent since it is reported that while alginate does not interact with BSA, chitosan does. The low protein interaction for the films can be explained by the fact that the layers in a LbL multilayer are not completely structured and there is a certain degree of interpenetration between subsequent layers. For very thin films, although chitosan may be the outer layer, alginate chains may be protruding from the film and reduce the protein attachment [37].

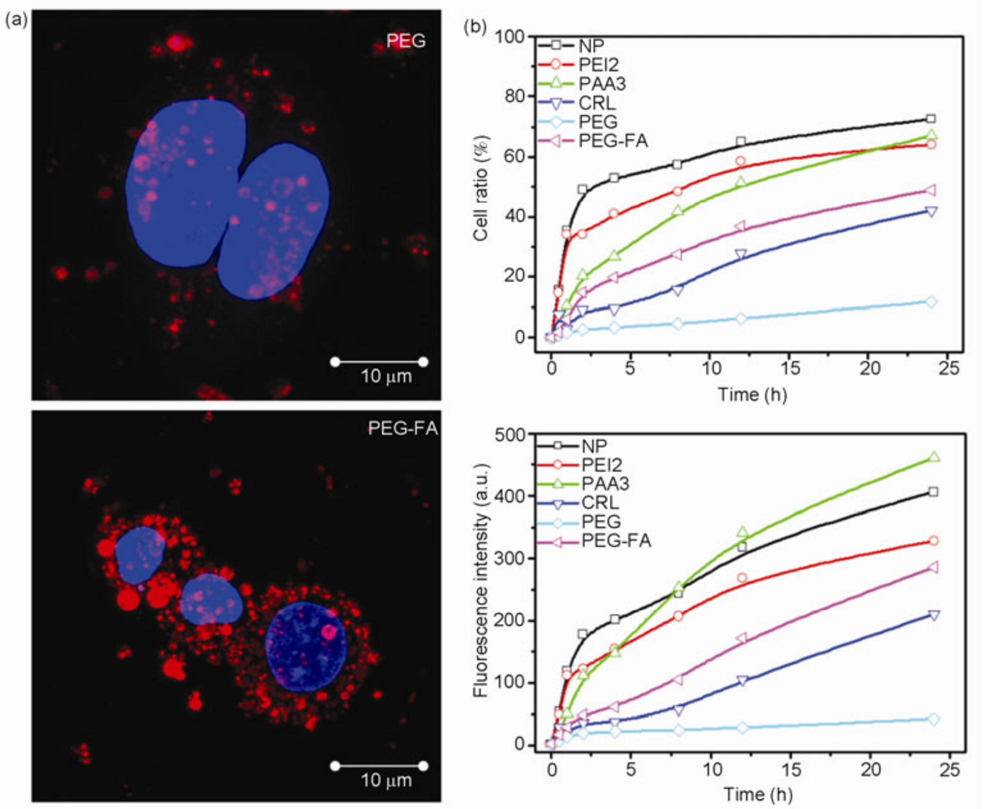

**Figure 2** (a) CLSM images of HepG2 cells exposed to PEGylated and PEGylated folic acid modified by PLGA NPs. Both PEG and PEG/FA were attached to PEI/PAA films. PLGA NPs were labelled by encapsulation of Rhodamine B. The cell nucleus was stained with DAPI for visualisation. The confocal images show that the amount of labelled NPs per cell is significantly lower for PEG coated NPs than for PEG FA as was expected. (b) from FACS measurements, the cell ratio calculated as the number of cells showing fluorescence over a certain threshold to the total number of cells and the maximum fluorescence intensity of the intensity distribution curve of HepG2 cells exposed to PLGA NPs, PLGA NPs with PEI/PAA, PLGA NPs with PEI/PAA and PEG, and PLGA NPs with PEI/PAA and PEG/FA. Both uptake and fluorescence intensity are higher for the PLGA NPs and PLGA NPs with PEI/PAA than with those with PEG. Instead, the coating with PEG/FA increases both over the values obtained for PEG alone. Reproduced from Ref. [35].

## 4 LbL in combination with lipid assembly for the targeting of NPs

Following the strategy of applying LbL as a means to engineer NPs in Romero *et al.* [38], the coating of colloidal particles previously LbL engineered or polyelectrolyte capsules with lipid membranes offer many possibilities to generate a biocompatible coating or to avoid unspecific interactions between the capsules and biomolecules, as well as to prolong circulation of drug carriers.

Besides these applications, the deposition of lipid vesicles composed of 1,2-dioleoyl-sn-glycero-3-choline (DOPC) and 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS) on top of PLGA NPs coated with a LbL film results in a means to increase the uptake of NPs by the cells and to target the NPs towards the nucleus and endoplasmic reticulum (ER). Coating with lipids provides a simple route to control NP uptake in cells as well to target the delivery to different intracellular regions while avoiding covalent chemistry and the use of specific receptors. The NPs that seem to translocate preferentially in the ER, could have applications for the treatment of antiviral human pathogens, such as human immunodeficiency virus (HIV), hepatitis C virus (HCV) or hepatitis B virus (HBV), and also in other diseases such as anticancer vaccinations.

Lipid assembly on top of the PLGA NPs takes place through electrostatic interactions since lipids are charged. Once assembled on top of the LbL film, the lipids spread and fuse forming a continuous layer [38, 39]. As for the other coating, cellular uptake and also the uptake pathway of the PLGA NPs were followed by FACS and CLSM using the HepG2 cell line [40]. The confocal images in Figure 3 show the co-localisation of the NPs with the ER as a function of their surface modification. As can be seen from the images there is no spatial overlapping between the fluorescence coming from the labelled ER and the labelled NPs (dyes were chosen with minimal spectral overlap), when these are not coated with the lipid. Co-localisation of the NP and the ER increases as the amount of charged lipid in the lipid coating decreases. In parallel, it can be seen in the images that as the percentage of charged lipids decrease the NPs tend to form larger aggregates that place close to the nucleus.

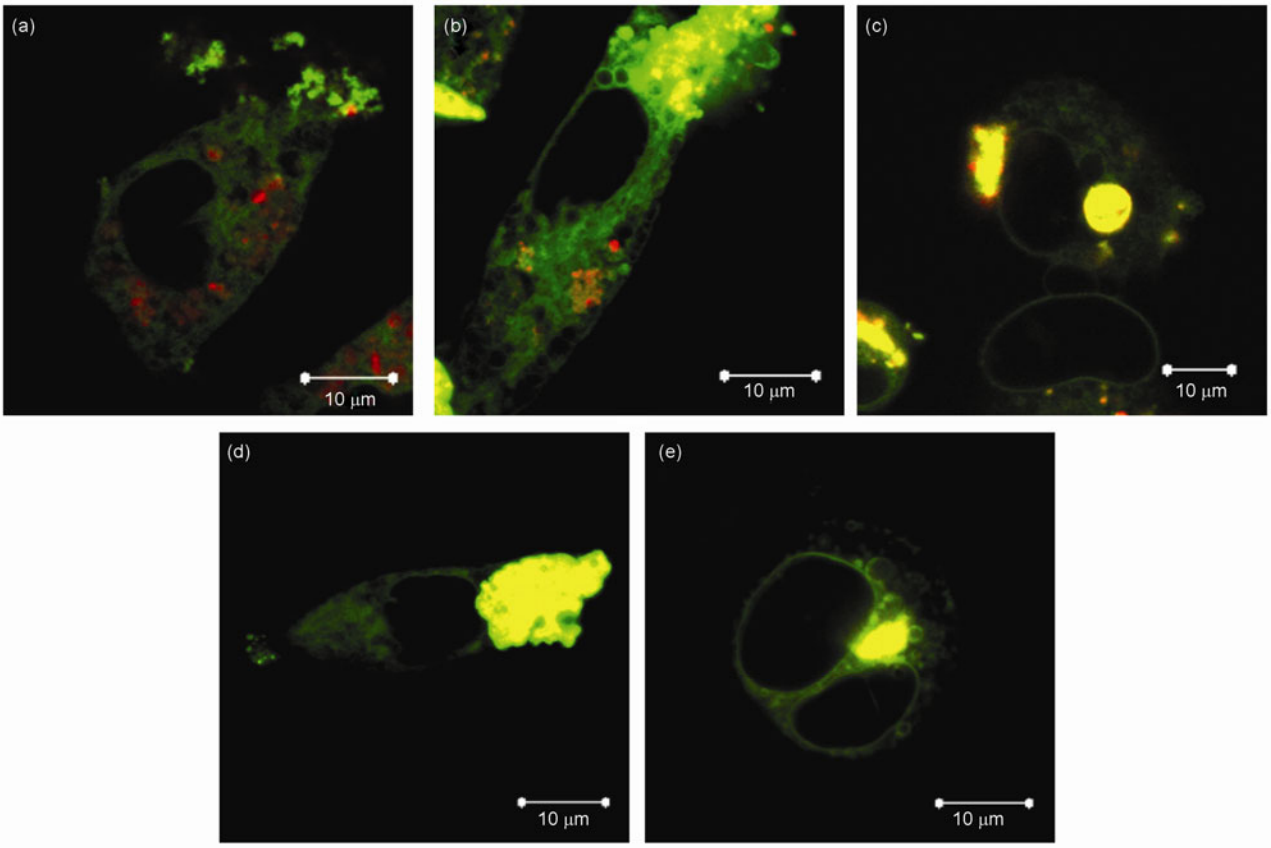

**Figure 3** CLSM images of HepG2 cells with stained ER (green) after co-culture with (a) PEI stabilised PLGA NPs (red) and lipid coated PLGA NPs (red) with lipid molar ratios DOPC/DOPS: (b) 65 : 35, (c) 75 : 25, (d) 85 : 15 and (e) 95 : 5 incubated for 12 h. Reproduced from Ref. [38].

## 5 Layer by layer engineered nanoparticles for the sustainable delivery of antibodies and nucleotides

Although the encapsulation of an antibody or DNA/RNA inside PLGA would be possible, since these molecules can be considered as medium or large macromolecules their release would depend on the degradation of the NP, being a slow process of several days or even weeks. An alternative is to carry the antibody on the surface of the NPs. LbL provides a means to deposit the therapeutics on top of the PLGA without their chemical modification.

The amount of therapeutic to be encapsulated, the particle dose, can be controlled by the number of assembly steps on the nanoparticles. Since delivery must take place, the layers should not be fully stable and should peel off over time. For antibodies, this should occur extracellularly for their blocking action on receptors, while in the case of nucleotides the delivery must take place intracellularly.

In Romero *et al*. [41], LbL has been applied for the encapsulation of antiTNF-α antibody on top of PGLA NPs. This antibody binds to the tumour necrosis factor alpha (TNF-α), thereby down regulating inflammatory reactions in autoimmune diseases [42]. The encapsulation of the antibody inside the PLGA NPs was avoided since due to the size of the antibody its release would only take place when the NPs are degraded as already mentioned. For the assembly of the antiTNF-α, proper conditions were determined such that the release of the antibody takes place progressively and avoiding a burst release or instantaneous decomposition of the multilayer in cell media. Complexation of antiTNF-α with an excess of alginate and the assembly of the complex as a negatively charged component in the multilayer proved to be the best means to achieve sustained delivery of the antibody [41].

The release of antiTNF-α was studied in PBS and in the presence of HepG2 cells. This cell line was chosen as it has been used previously as an *in vitro* model to study hepatic inflammation and injury caused by TNF-α in ethanol induced damage [43]. The amount of antiTNF-α present in PBS and the HepG2 cell suspension after co-incubation with PLGA NPs was quantified with a specific kit for antiTNF-α detection, the Q-ADA Kit [41]. In Figure 4(a) the amount in ng/mL of antiTNF-α released from the NPs in a population of one million cells has been displayed as a function of the incubation time for both, PLGA NPs with 11 and 25 layers of poly (lysine) and the alginate/antiTNF-α complex. In Figure 4(b) the release ratio of antiTNF-α from the PLGA NPs has been plotted as a function of time. We can observe that in both cases, 11 or 25 layers, the increase in concentration of antiTNF-α in HepG2 cells resembles a first order release profile. Similar release profiles were obtained in PBS.

When HepG2 cells were exposed to PLGA NPs coated with 11 layers, a gradual increase of the antibody inside $10^6$ cells over time is shown. After 4 days of co-incubation, the antibody uptake reaches 22 ng/mL, which represents 80 % of the encapsulated antibody. For the cells exposed to PLGA NPs coated with 25 layers, a burst release of the antibody is observed at the beginning of the profile. The release of antiTNF-α (ng/mL) after 4 days represents 95 % of the antibody encapsulated for the given sample of PLGA NPs. The encapsulation of antiTNF-α in the multilayer coating onto PLGA NPs provides a means for the gradual release of the antibody.

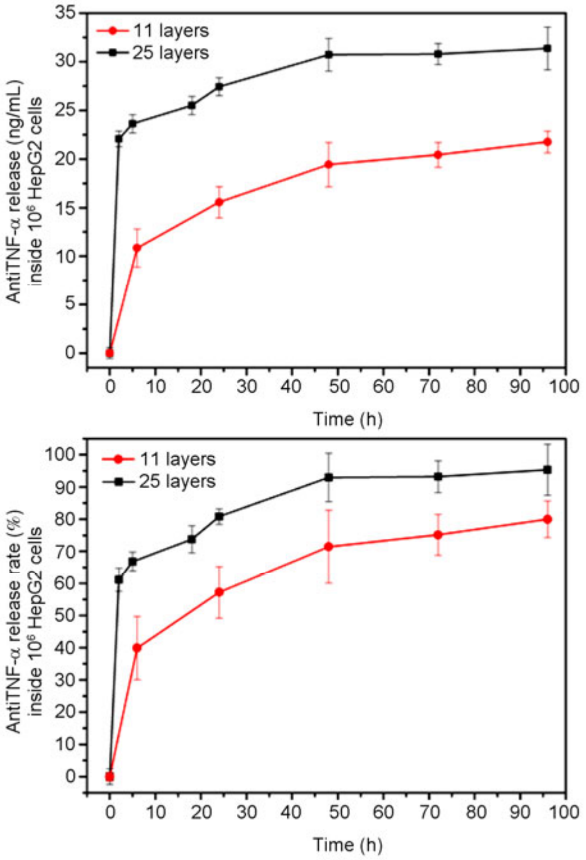

**Figure 4** Release profiles of antiTNF-α liberated in a suspension of $10^6$ HepG2 cells from the 11 and 25 LbL coatings of PLGA NPs. The release profiles are represented for concentration in ng/mL (a) and released ratio (b). Reproduced from Ref. [41].

## 6 Multilayers for controlled release of encapsulated molecules

### 6.1 Release behaviour of fluorescent dyes from PLGA nanoparticles

Multilayers on top of PLGA particles form a thin film with multiple charges and as such, depending on how permeable the resulting assembled film is, this can become a barrier for the release of encapsulated molecules. Since multilayers have multiple charges, both positive and negative, if the encapsulated molecules are charged they can interact with the charges of the multilayer, which may contribute to their retention in the film altering their release. Zhou *et al.* [43] investigated the release behaviour of 3 fluorescent dyes (Rhodamine 6G (Rh6G is positive in PBS), Fluorescein (almost neutral in PBS), and 5(6)-Carboxyfluorescein (negative in PBS)) incorporated into PLGA NPs, coated in PEI/PAA. The release behaviour of the incorporated dyes was investigated at two distinct temperatures, 37 and 60 °C, to mimic physiological conditions and temperatures higher than the glass transition temperature ($T_g$) of PGLA respectively. Above the $T_g$ the polymer chains in PLGA are more mobile and should favour the release of encapsulated molecules. Fluorescent dyes are used here as model systems for drugs. At the lower temperature, the release curves showed that the release behaviour of all the PLGA NPs with the numbers of multilayers could be well fit with a zero order release curve, and that the release rate was much lower when PAA was the outermost layer of the NPs, and with increasing number of layers (see Figure 5).

The release rates for Rh6G were much lower when PAA was the outmost layer (~0.009–0.01 $d^{-1}$), approximately 50–65% of the values for PEI (~0.005–0.0067 $d^{-1}$). The slow rate of release of Rh6G at 37 °C is probably due to the hydrophobic character of the dye 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 complexation of the negatively charged PAA with the positively charged Rh6G, which must diminish diffusion from the NPs.

At high temperature, chosen to be higher than the $T_g$ of the PLGA (55–58 °C), release experiments showed that all the dyes can be released, there is a very prominent burst profile observed in the first 2 hrs for Rh6G and Fluorescein that is less pronounced in the case of 5(6)-carboxyfluorescein. The profiles are shown in Figure 6.

In contrast to the glassy state in which the polymer chains are hardly mobile, above $T_g$ the segments of the PLGA chains have greater freedom to move, leading to faster diffusion of dye molecules in the polymer matrix [44, 45], leading to higher initial release rates. The slower release of 5(6)-carboxyfluorescein can be explained in terms of charge interaction between the dye and the PEI layers which retards the release of 5(6)-carboxyfluorescein. However, no interaction, or even repulsion, appears to exist between the PEI and the Rh6G and fluorescein dyes, leading to faster release.

The positively charged Rh6G binds to the multilayers to a larger extent when PAA is the outermost 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 the charge of the outer layer very small.

## 6.2 Intracellular release behaviour of doxorubicin encapsulated PLGA NPs

Doxorubicin (Dox) is one of the most frequently used drugs for cancer treatment and is often used as a model drug for delivery studies due to its intrinsic fluorescence which, makes it suitable for use with several fluorescence characterisation techniques. Nevertheless, typical fluorescence techniques, such as FACS or CLSM, are frequently limited when applied to intracellular characterisation of Dox release from a carrier. This is due to the difficulty associated with distinguishing between fluorescence due to the released drug and that from the encapsulated drug. Dai *et al.* [46], showed for the first time that fluorescence Lifetime Imaging Microscopy (FLIM) could be applied to measure doxorubicin (Dox) delivery inside cells and permitted the

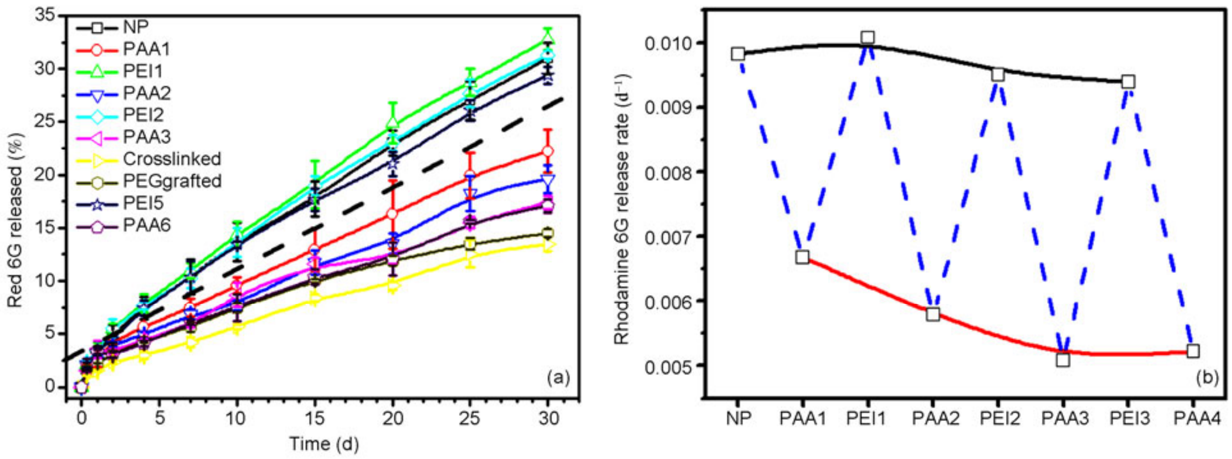

**Figure 5** Release profiles of PLGA nanoparticles with rhodamine 6G at 37 °C, plotted against time for different multilayer coatings (a) and plotted as a function of layer number versus release rate (b). Reproduced from Ref. [43].

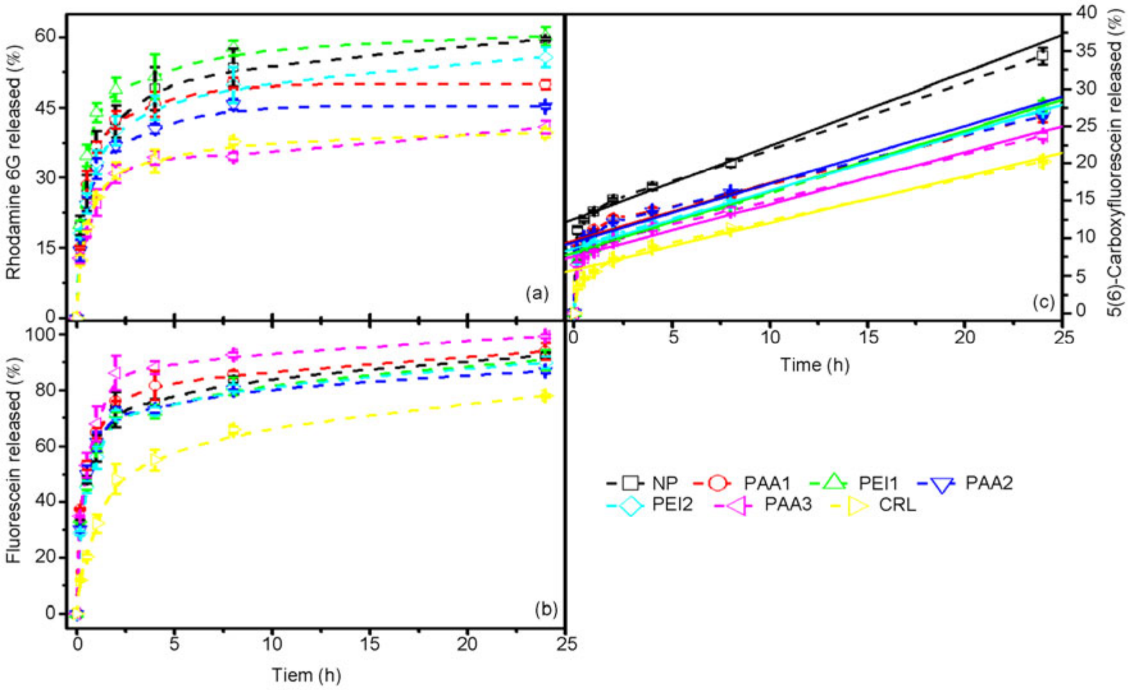

**Figure 6** Release profiles of nanoparticles with rhodamine 6G (a), fluorescein (b) and 5(6)-carboxyfluorescein (c) at 60 °C, plotted against time for different multilayer coatings. Reproduced from Ref. [43].

local environment of the drug inside the cell to be probed.

Romero *et al*. investigated the effectiveness of PLGA NPs, fabricated in our laboratory, for the intracellular release of Dox [47]. Following the preparation of the Dox encapsulated PLGA NPs, the release of Dox from NPs was first studied in PBS, with the kinetics of the NPs and Dox uptake "*in vitro*" then measured via FACS. Finally, Dox release was measured at the single cellular level, via FLIM, in HepG2 cells.

As in the study by Zhou *et al*. [43], the release behaviour of Dox was characterised at two temperatures, 37 and 60 °C, again to mimic physiological conditions and temperatures greater than $T_g$. Similar release profiles were seen, with a more rapid release at the higher temperature (Figure 7). Flow cytometry showed that PLGA NPs and Dox followed the same initial kinetics. However, after approximately 8 h of incubation, the fluorescence intensity of the Dox significantly reduced indicating the release of Dox to either the media or to the cell (Figure 8).

Prior to studying the intracellular release of Dox from PLGA NPs the Dox loaded PLGA NPs were characterised via FLIM and found to have a lifetime of 3.12 ns. Dox in water was also characterised via FLIM and determined to have a lifetime of 1.15 ns. As the release profile of PLGA NPs is relatively rapid in the initial stages (Figure 7), the HepG2 cells were incubated for only 4 h. FLIM studies showed that the fluorescence lifetime of Dox inside the cell has a value of 1.61 ns, shorter than that measured inside the PLGA NPs (3.12 ns) and longer than that in water (1.15 ns),

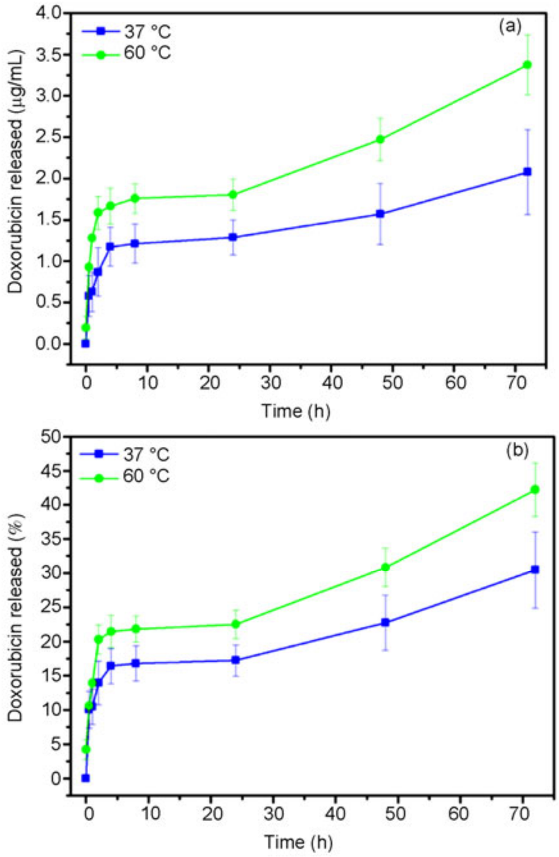

**Figure 7** Release profiles of doxorubicin from PLGA NPs in PBS at 37 and 60 °C, plotted for (a) μg mL$^{-1}$ of doxorubicin released and (b) percentage of doxorubicin released, from 1 mg of PLGA NPs in PBS. Reproduced from Ref. [47].

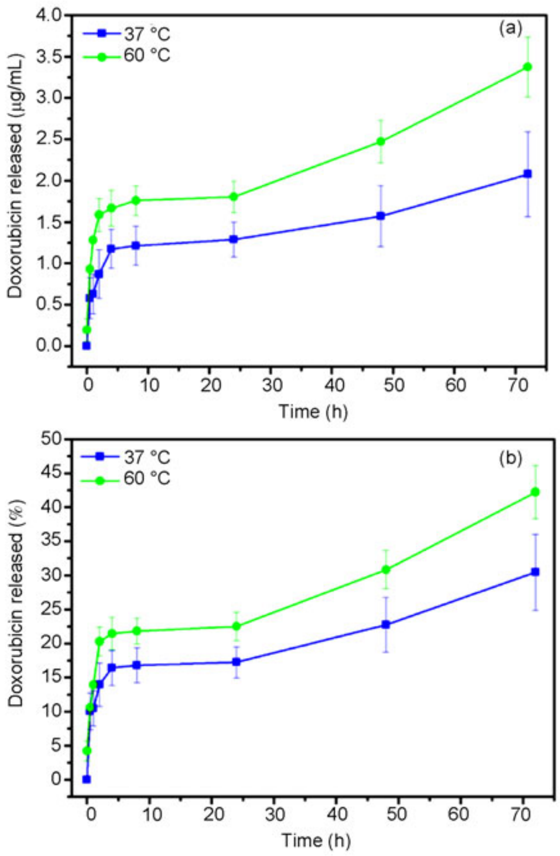

**Figure 8** (a) Fluorescence intensity of PLGA NPs and Dox inside HepG2 cells and (b) percentage of doxorubicin and PLGA NPs taken up by HepG2 cells. Reproduced from Ref. [47].

see Figure 9. The lifetime of Dox obtained inside the cytoplasm of HepG2 cells is comparable with the lifetime value obtained by Dai *et al.* in the cytoplasm of HeLa cells [6]. The results obtained suggested that after 4 h of incubation, Dox is fully released from the PLGA NPs and preferentially locates in the cell cytoplasm. No measureable lifetime could be measured in the cell nucleus.

These results indicate the usefulness of PLGA as a drug delivery vehicle with tunable release properties that can be easily controlled based upon the number of layers and the ratio of the polymer used in fabricating the PLGA NPs.

## 7 Conclusions and perspectives

The LbL assembly of polyelectrolyte provides a robust tool for surface modification that can be employed in the engineering of NPs, like PLGA, for their application in drug delivery, taking advantage of their ease of application, which basically requires only two polyelectrolytes of opposite charge. Indeed, we have shown throughout this article the numerous possible applications of LbL toward the engineering of PLGA NPs with a drug delivery scope, as a means to control cell uptake, intracellular localisation, to restrict unspecific interactions, as barriers for controlled delivery of encapsulated molecules and as a means to encapsulate large molecules located between layers.

The combination of LbL with covalent chemistry and biological self assembly has been used to tune cell uptake and direct the NPs intracellularly. The LbL coating formed upon the assembly of at least two charged polyelectrolytes offers multi-functional groups associated to the monomers of the polyelectrolytes. Using polyelectrolytes with amine or carboxylate groups we have been able to apply simple condensation chemistry and attach PEG and folic acid to the NPs. The LbL technique prevents the NP stability from being affected and also makes it possible to functionalise any NP despite the chemistry of their capping agent, provided that they are charged. Other molecules could be attached to the NPs following this strategy such as Trojan peptides, antibodies, carbohydrates that could render specific recognition properties to the NP or prevent unspecific interactions.

The LbL coating can itself be used to prevent unspecific interactions when composed of charged carbohydrates with limited protein interactions. The carbohydrate coating can also be used for the covalent attachment of folic acid or other molecules bearing recognition properties while avoiding Pegylation.

Self assembly of lipids on top of the polyelectrolyte cushions have been shown to direct the NPs intracellularly and influence uptake depending on their composition, i.e. percentage of charged and zwitterionic lipids. The lipid layers could also be a starting point for the assembly of other biomolecules that need a lipid environment as a support such as viral nanoparticles or the recently developed virosomes. This strategy is presently being addressed in our laboratory with the aim of endowing NPs with the recognition properties of viruses and viral uptake mechanisms.

We have also seen LbL coatings being employed for the encapsulation of antibodies between polyelectrolyte layers. The effectiveness in down regulating inflammatory processes in the case of antiTNF-α must be proved. Further work must be undertaken for specific applications and “*in vitro*” experiments must be followed by “*in vivo*” work. Also, the methodology developed is presently being extended in our group to other antibodies and to gene therapeutic agents: plasmids, siRNA.

The impact of the multilayers in the release of drugs encapsulated in the colloidal particles requires a deeper study taking into account the hydrophobic character of the drugs and their size. Initial work has been performed on studying the release of encapsulated drugs intracellularly and will be extended to NPs coated with multilayers.

The use of LbL coatings for the engineering of NPs for drug delivery offers multiple possibilities, as well as challenges. There is plenty of work that still required to be done to fabricate systems with specific recognition,

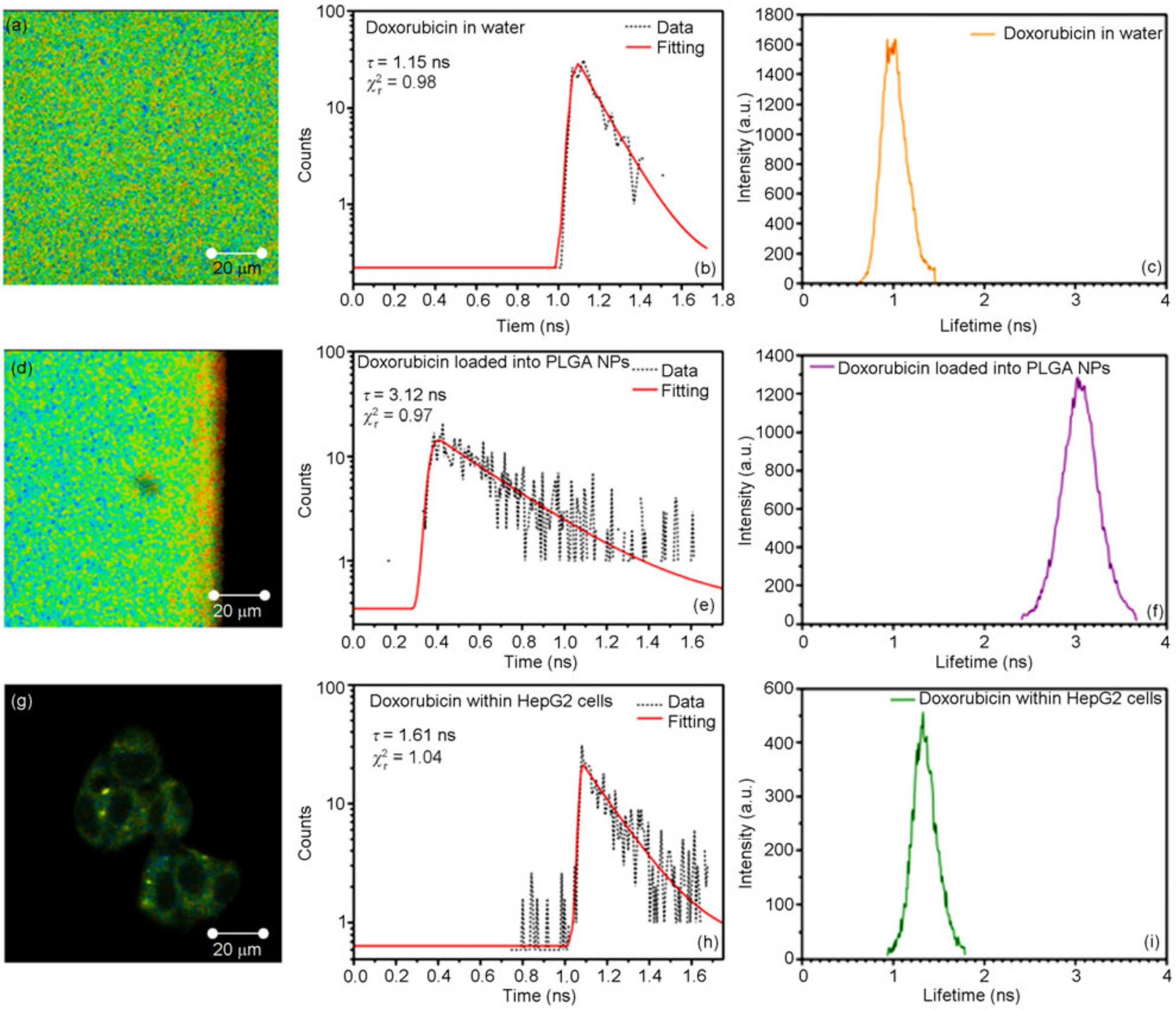

**Figure 9** Lifetime images and corresponding lifetime decay curves and lifetime distributions of Dox in water (a–c), Doxorubicin loaded into PLGA NPs (d–f) and HepG2 cells incubated with doxorubicin loaded PLGA NPs (g–i). Reproduced from Ref. [47].

selection and delivery properties for the treatment of specific diseases, and more importantly to translate this research to the "*in vivo*" environment.

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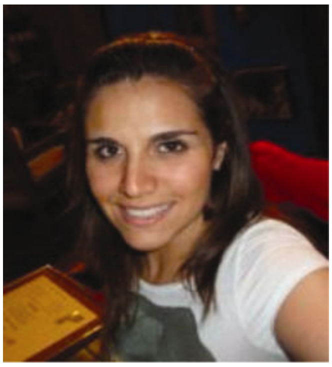

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