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Impact of PEG and PEG-*b*-PAGE modified PLGA on nanoparticle formation, protein loading and release

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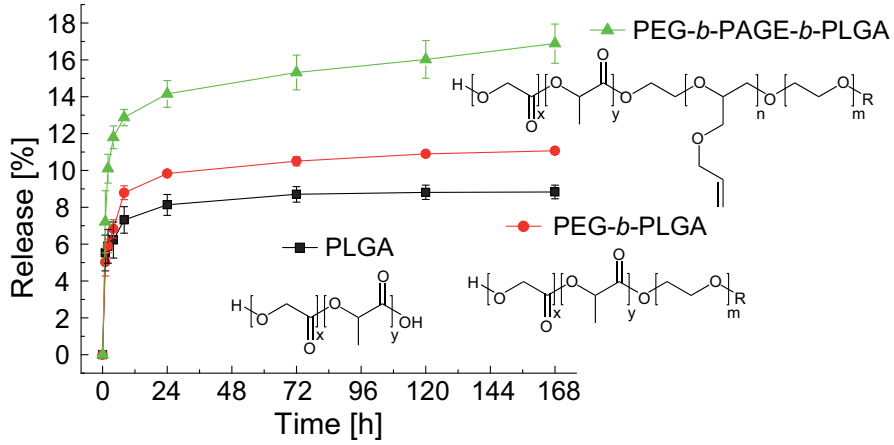
Graphical abstract



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ABSTRACT

The effect of modifying the well-established pharmaceutical polymer PLGA by different PEG-containing block-copolymers on the preparation of ovalbumin (OVA) loaded PLGA nanoparticles (NPs) was studied. The used polymers contained poly(D,L-lactic-*co*-glycolic acid) (PLGA), polyethylene glycol (PEG) and poly(allyl glycidyl ether) (PAGE) as building blocks. The double emulsion technique yielded spherical NPs in the size range from 170 to 220 nm (PDI < 0.15) for all the differently modified polymers, allowing to directly compare protein loading of and release. PEGylation is usually believed to increase the hydrophilic character of produced particles, favoring encapsulation of hydrophilic substances. However, in this study simple PEGylation of PLGA had only a slight effect on protein release. In contrast, incorporating a PAGE block between the PEG and PLGA units, also eventually enabling active targeting introducing a reactive group, led to a significantly higher loading (+ 25%) and release rate (+ 100%), compared to PLGA and PEG-*b*-PLGA NPs.

ABBREVIATIONS

API	Active pharmaceutical ingredients
BCA	Bicinchoninic acid protein
Da	Dalton
DCM	Dichloromethane
DLS	Dynamic light scattering
EE	Encapsulation efficiency
L	Loading
Mn	Molar masses
Mw	Molecular weight

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NP	Nanoparticle
OVA	Ovalbumin
PAGE	Poly(allyl glycidyl ether)
PEG	Polyethylene glycol
PLGA or P	Poly(D,L-lactic-*co*-glycolic acid)
PB	Phosphate buffer
PBS	Phosphate buffer saline
PDI	Polydispersity index
PP	PEG-*b*-PLGA
PPP	PEG-*b*-PAGE-*b*-PLGA
PQ	Partitioning coefficient
PVA	Polyvinyl alcohol
RB	Rose Bengal
SEM	Scanning electron microscopy

KEYWORDS

Poly(D,L-lactic-*co*-glycolic acid), polyethylene glycol, poly(allyl glycidyl ether), ovalbumin, protein delivery, drug delivery

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1. INTRODUCTION

It is well-known that larger, complex and hydrophilic active pharmaceutical ingredients (API) such as proteins, administered without any protection/vehicle, are prone to degradation and suffer from a low capability to cross biological barriers. To overcome this problem the incorporation of proteins in the polymeric matrix of nanoparticles (NPs) appears to be a promising strategy (Barratt, 2003; Bramwell and Perrie, 2005; Csaba et al., 2006; Santander-Ortega et al., 2010). Numerous advanced drug delivery systems, such as NPs, microparticles and liposomes were developed in the last decades (Soppimath et al., 2001; Yadav et al., 2011). Among those, NPs show high potential to control the delivery of various APIs (Conti et al., 1991; Sanders et al., 1984). Many polymeric matrix materials have been intensively investigated in this context, including poly(D,L-lactic-*co*-glycolic acid) (P, PLGA) (Dillen et al., 2004; Fonseca et al., 2002; Govender et al., 1999; Lemoine and Préat, 1998; Nafee et al., 2007), chitosan (Janes et al., 2001; Prabha et al., 2002; Rhim et al., 2006), poly-ε-caprolactone (Gan et al., 1999; Leroueil-Le Verger et al., 1998), starch (Kreuter, 1991; Le Corre et al., 2010), alginate (Johnson et al., 1997; Sarmento et al., 2006) and gelatin (Balthasar et al., 2005; Khan and Schneider, 2013; Truong-Le et al., 1999). One of the most common biodegradable and biocompatible polymers (Langer and Peppas, 1981) used to prepare NPs for the encapsulation of proteins, via the water-in-oil-in-water (w/o/w) double emulsion method, is PLGA. The major disadvantages of using a hydrophobic polymer such as PLGA are however a limited capability of both protein loading and release over time. There has been an increasing interest in the past years to overcome this problem through the development of more hydrophilic PLGA derivatives, as an increased hydrophilic character is expected to improve interaction with and facilitate encapsulation of hydrophilic substances such as proteins. In this respect, the covalent linkage of polyethylene

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glycol (PEG) as a flexible, hydrophilic, and FDA-approved molecule to PLGA (Dumitriu and Popa, 2013) has been extensively described in literature (Gref et al., 2000; Liu et al., 2010). The PEGylation of PLGA, in order to produce PEG-*b*-PLGA (PP), also increases the circulation time of the NPs in the human body, as a consequence of passively masking the NPs from the host immune system (Bazile et al., 1995). The next step in the development of a superior PLGA is considered to be the addition of a functional block to PEG-*b*-PLGA, allowing for the covalent linkage of targeting ligands for active drug targeting/delivery. This is a new approach based on the insertion of a poly(allyl glycidyl ether) (PAGE) group between PEG and PLGA units of PEG-*b*-PLGA (PEG-*b*-PAGE-*b*-PLGA) (Figure 1). This PAGE group can then act as a linker for drug targeting ligands. In the current study the influence of hydrophilic PEG building blocks and different subtypes of PLGA on the properties of various PEG-*b*-PLGA and non-functionalized, novel PEG-*b*-PAGE-*b*-PLGA (PPP) polymers was systematically investigated, while keeping all other method parameters fixed – an approach which has not been extensively explored in literature to date. The produced PEG-*b*-PLGA and PEG-*b*-PAGE-*b*-PLGA polymer subtypes had different lactic-to-glycolic acid ratios, different molar masses (Mn) of PLGA and different degrees of PEGylation. The evaluation of the effect of various building blocks on NP characteristics was based on measurements of protein encapsulation efficiency, loading and release. For this purpose, the model protein ovalbumin (OVA), found in egg white, was used for all experiments. OVA is commonly used as a model antigen/protein in murine animal studies investigating protein drug delivery with hydrophobic micro- or nanoparticles; its use in the current work therefore allowed for comparison of the obtained data to literature (Reddy et al., 2007; Slütter et al., 2009). Further to the evaluation of encapsulation, loading and release, the native structure of the protein within the different PLGA polymer NPs was studied. For a meaningful comparison of polymer effects it was important to identify a standard NP preparation procedure and to keep all other preparation parameters identical. The optimization of such a preparation procedure was performed for the PEG-*b*-PAGE-*b*-PLGA polymer and adapted to the other polymers. Protein

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release was investigated over the course of 7 days, in order to allow for evaluation of both short and long-term release kinetics.

2. MATERIALS AND METHODS

2.1. MATERIALS

Different types of PLGA (Resomer®, Table 1) were supplied by Evonik Industries AG (Darmstadt, Germany). Various PEG-*b*-PLGA and PEG-*b*-PAGE-*b*-PLGA polymers were synthesized in the Laboratory of Organic and Macromolecular Chemistry (IOMC) and Jena Center for Soft Matter (JCSM), at the Friedrich Schiller University of Jena (Table 1) (Justyna A. Czaplewska, Tobias C. Majdanski, Markus J. Barthel, Michael Gottschaldt, 2015). The number average molar mass (Mn) of the different polymers was given as a mean average, determined by size exclusion chromatography and nuclear magnetic resonance. Albumin from chicken egg white (ovalbumin, type Grade V, purity ≥ 98%, Mw = 44 kDa), the QuantiPro™ BCA Assay Kit (for 0.5-30 µg/ml protein), and D-(+)-trehalose dihydrate (from *Saccharomyces cerevisiae*, purity ≥ 99%), dichloromethane (DCM, HPLC grade) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany), as were sodium chloride (NaCl, BioXtra, purity ≥ 99.5%), potassium chloride (KCl, BioXtra, purity ≥ 99.0%), sodium phosphate dibasic heptahydrate (Na_2HPO_4 * 7H2O, meets USP testing specifications) and monobasic potassium phosphate (KH_2PO_4, for molecular biology, purity ≥ 98.0%), which were used to prepare phosphate buffer (PB Buffer) and phosphate buffered saline (PBS buffer). Polyvinyl alcohol (PVA) was purchased from Kuraray Europe GmbH (Hattersheim, Germany). Water used for all preparations and investigations was produced by a Millipore Q-Gard 2 purification system (Merck Millipore, Billercia, United States). All other used chemicals were of analytical grade.

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2.2. PREPARATION OF NANOPARTICLES

OVA-loaded NPs based on PLGA, PEG-*b*-PLGA and PEG-*b*-PAGE-*b*-PLGA were prepared using a double emulsion solvent evaporation technique. Briefly, 50 mg of polymer was dissolved in 2.5 ml DCM (20 mg/ml) and emulsified with 0.5 ml OVA solution (in PBS pH 7.4, OVA concentration 20 mg/ml) by sonication with 35% amplitude (energy introduced 280 J) at room temperature (Branson digital sonifier 250, Danbury, United States), to obtain a w/o emulsion. Subsequently 5 ml of a 2% PVA solution (w/v) was added to the first emulsion and sonicated again with 35% amplitude (energy introduced 595 J), at room temperature, to obtain the w/o/w emulsion. The same procedure was carried out with only 0.5 ml PBS in the absence of OVA solution to prepare blank, unloaded polymer NPs. The double emulsion was diluted by adding water dropwise followed by stirring at 1,000 rpm overnight at room temperature, to evaporate the DCM. To produce NPs surface coated with OVA, unwashed blank NPs after overnight DCM evaporation were incubated for 1 h with 0.5 ml OVA solution (in PBS pH 7.4, OVA concentration 20 mg/ml). Finally, unloaded, OVA-loaded and OVA surface-coated NPs were purified, by centrifugation at 15,000 g for 11 min (Rotina 420 R, Hettich Lab Technology, Tuttlingen, Germany) and washed twice with water. After purification trehalose and PVA were added to NPs as a cryoprotectant and stabilizer for lyophilization respectively. For lyophilization the NP suspension was frozen for 3 h at -80 °C and lyophilized for 3 days under vacuum (Alpha 2-4 LSC, Christ GmbH, Osterrode, Germany). Four batches of every formulation were prepared.

2.3. CHARACTERIZATION OF NANOPARTICLES

The average size (in nm), size distribution (polydispersity index - PDI) and ζ-potential (in mV) of the polymeric NPs were measured using dynamic light scattering (DLS) and electrophoretic mobility (Zetasizer Nano ZSP, Malvern Instruments, Herrenberg, Germany) at 25 °C. The measurements were performed with aqueous dispersions of NPs (~ 0.1 mg/ml) prior to lyophilization. The surface morphology of NPs was determined by scanning electron microscopy

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(SEM - Zeiss EVO HD15, Jena, Germany). NPs were coated with a gold layer of approximately 10 nm under vacuum before SEM examination (accelerating voltage 5 kV, focal distance 10 mm).

2.4. DETERMINATION OF ENCAPSULATION EFFICIENCY AND LOADING

A bicinchoninic acid protein (BCA) assay kit (QuantiPro™ BCA Assay Kit) was used for the determination of encapsulation efficiency (% EE = encapsulated OVA [mg]/ initial drug in formulation [mg] * 100) and loading (% L = encapsulated OVA [mg]/ weight prepared NPs [mg] * 100) of OVA in NPs, according to the manufacturer's instructions. Accordingly, 1 mg of lyophilized NPs was dissolved in 1 ml of 1 M NaOH solution (1 mg/ml). After overnight incubation and neutralization with 1 M HCl at 20 °C, the protein concentration within dissolved NP samples was determined using the QuantiPro™ BCA Assay Kit and the Tecan Infinite M200 Pro (Männedorf, Switzerland) plate reader at λ= 562 nm. Blank NPs were used as control. Each batch was analyzed in triplicate.

2.5. IN VITRO RELEASE OF PROTEIN

Approximately 10 mg of lyophilized NPs were incubated with 10 ml of PBS buffer (100 mM and pH 7.4) in glass vials, over 7 days under continuous stirring at 150 rpm at 37 °C. At appropriate time intervals (1 h, 2 h, 4 h, 8 h, 1 d, 3 d, 5 d, 7 d) the samples were collected and centrifuged for 15 min at 20,000 g. The clear supernatant was used to determine the amount of OVA released from the formulation using the QuantiPro™ BCA Assay Kit. Each batch was analyzed in triplicate.

2.6. ACTIVITY OF OVA

Quantification of the activity of OVA encapsulated or coated on the surface of the NPs was performed using an ELISA assay, in order to determine whether the native state of the protein was maintained. The prepared NP dispersion (1 mg/ml) was incubated for 24 h in PBS buffer

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pH 7.4 at 37 °C under continuous stirring at 150 rpm. The dispersion was centrifuged at 20,000 g for 15 min and the total amount of protein in the supernatant was determined using the QuantiPro™ BCA Assay Kit. To determine the amount of active OVA in the same supernatant an ELISA assay (Serazym® Ovalbumin ELISA, Seramun Diagnostica GmbH, Heidesee, Germany, range 0.625-20 ng/ml, lower limit of detection, LLOD = 0.125 ng/ml), which was performed according to the instructions provided by the manufacturer, was used. Consequently, the amount of OVA quantified with the ELISA assay was divided by the total amount of OVA within the supernatant to determine the percentage of active or so-called native protein (% Activity = (amount of native OVA [mg]/ total amount of OVA in the supernatant [mg]) * 100). As negative control supernatant from blank particles was employed, and a freshly prepared OVA solution in PBS was used as positive control.

2.7. DETERMINATION OF SURFACE HYDROPHOBICITY

The surface hydrophobicity was determined by the adsorption of the hydrophobic dye Rose Bengal (RB) on NPs. The hydrophobic dye (with a constant c_{RB} = 40 µg/ml) was incubated with an increasing NP concentration (0.3125-5 mg/ml) for 1 h at room temperature (Müller et al., 1997). After the incubation time the suspensions were centrifuged at 20,000 g for 15 min and the absorption of the supernatants was measured at λ= 542 nm. An RB calibration curve (40-0.625 µg/ml) was prepared to determine the RB concentration in the supernatant. The partitioning coefficient (PQ) of RB for each NP concentration was then calculated by dividing the adsorbed amount of RB on NP surface by the free amount of RB in the dispersion medium. This PQ was plotted versus the increasing NP surface area. The obtained plots were used a measure for the degree of NP surface hydrophobicity.

2.8. STATISTICAL ANALYSIS

Statistical analysis of loading and encapsulation efficiencies was performed via one way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test using GraphPad

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Prism 6 software (GraphPad Software, Inc, La Jolla, CA). The confidence interval was set at 95%, a p value of > 0.05 was taken as not significant (n.s.) and a p value of < 0.05 was accepted as significant (*); $p < 0.01$ (**), $p < 0.001$ (***).

3. RESULTS AND DISUSSION

In many studies to date, the influence of changing numerous process parameters - such as the amount of polymer and drug used, the time for preparation, and the selected excipients (different emulsifier or release modifier) - on particles produced via the double emulsion method has been described (Buske et al., 2012; Corrigan and Li, 2009; Cruz et al., 2011; Feczkó et al., 2011; Mundargi et al., 2008). However, for comparison of the potential of a polymer to act as an encapsulation material it is of utmost importance to keep the majority of process parameters unchanged. As a consequence, only the Mn of the different polymer building blocks, and hence the fraction of its influence on the polymers' properties, was varied.

3.1. CHARACTERIZATION OF NANOPARTICLES

Blank and OVA-loaded NPs of all PLGA subtypes were prepared using the same conditions by a double-emulsion method. The results yielded narrowly distributed (PDI < 0.15) NPs with a mean size of OVA-loaded PLGA, PEG-*b*-PLGA and PEG-*b*-PAGE-*b*-PLGA particles in the range of 170 - 220 nm (Figure 2). Blank particles were roughly 15-25 nm smaller than the OVA-loaded particles, which is not surprising taking into consideration the size of the encapsulated protein (mean size of a single OVA molecule determined by DLS is 2-3 nm). No substantial influence of PLGA, PEG or PAGE Mn or of lactic:glycolic acid ratio was found on NP physiochemical characteristics for any of the tested polymer derivatives (PLGA, PEG-*b*-PLGA and PEG-*b*-PAGE-*b*-PLGA). It should however be noted that NP could not be prepared from PEGylated PLGAs with molecular weight ratios of PEG to PLGA < 1:5 using the double emulsion method.

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All types of PLGA (Figure 2) were found to form particles with a negative surface charge of -29.1 ± 2.2 mV. Protein incorporation led to a reduction in magnitude of surface charge, to -20.1 ± 3.6 mV. No major difference in surface charge was observed for blank particles prepared from PLGA (-30 mV), PEG-*b*-PLGA (-29 mV) to PEG-*b*-PAGE-*b*-PLGA (-27 mV); this is due to the use of PVA, both as an emulsifier in the preparation method and most importantly, as a stabilizer for our final lyophilized product. The stabilization of the freeze-dried formulation with PVA was necessary to prevent particle aggregation. Therefore, the remaining PVA in the preparation acts to shield the NP interface and thus maintain a consistent zeta potential. The SEM images in Figure 2 show smooth, spherical NPs with sizes approximately 20 nm smaller (determined by Image J) than those measured by DLS. These differences are most likely due to conduction of SEM imaging in vacuum in contrast to DLS measurement of the hydrodynamic radius in suspension.

3.2. LOADING AND ENCAPSULATION EFFICIENCY

As mentioned, all NPs were prepared using the same conditions, with an initial fixed OVA concentration (20 mg/ml), what results in a maximum loading of 20% corresponding to 100% encapsulation efficiency. Encapsulation efficiency will therefore not be mentioned additionally during the results and discussion, with a focus placed instead on loading.

First the loadings of five commercially available PLGA types from Evonik (Figure 3) were determined. Three different Mn of PLGA (P 1 - 10,800 Da, P 2 - 23,200 Da and P 5 - 38,800 Da) were used to investigate the effect of different Mn on the loading while keeping the lactic:glycolic acid ratio of 50:50 constant. Furthermore, three PLGA types were used to show the influence of the lactic:glycolic acid ratio (P 2 - 50:50, P 3 - 65:35, P 4 - 75:25) on produced NPs, using the same Mn for PLGA of approximately 24,000 Da. For all PLGA polymers an average load of 8.2 ± 1.0%, independent of the Mn_{PLGA}, lactic:glycolic acid ratio and particle size was found.

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For the comparison of the three available PEG-*b*-PLGA polymers (PP 1-3) similar PLGA molar weights varying between 45,000 and 54,500 Da and constant lactic:glycolic acid ratio of 50:50 were used. The degree of PEGylation of polymers however increased, from 2.8% (PP 1) to 4.4% (PP 2) to 6.8% (PP 3). For the available PEG-*b*-PLGAs an overall average loading of 8.7 ± 1.0% was determined. Ultimately no significant increase of loading was encountered from lower to higher degree of PEG-*b*-PLGA PEGylation. Comparing PEG-*b*-PLGA NPs with the unmodified PLGA NPs a slight increase of the mean values for loading and encapsulation was observed, however this was not significant - an observation which has already been described in literature (Li et al., 2001; Tobío et al., 1998).

The major group of interest for investigation was the triblock polymer, PEG-*b*-PAGE-*b*-PLGA, assuming PLGA as a monoblock. For this polymer different combinations of PEGylation and Mn of PLGA were used, while maintaining a low variation of incorporated PAGE groups and keeping the lactic:glycolic acid ratio constant (Table 1). Overall an average loading for PEG-*b*-PAGE-*b*-PLGA NPs of 10.1 ± 3.0% was observed. This is a first hint that a combination of PEG and PAGE with PLGA acts to increase protein encapsulation, compared to PLGA and PEG-*b*-PLGA. We will first focus on the polymers PPP 1, PPP 2 and PPP 3 (Figure 3) with a constant PEG and PAGE fraction, but increasing Mn of PLGA from about 19,000 to 54,000 Da. The data show a significant increase of loading from 7.7 ± 0.5% (PPP 1) up to 11.4 ± 0.7% (PPP 3). From this group we can conclude that the loading increases with increasing Mn of PLGA in PEG-*b*-PAGE-*b*-PLGA NPs. With this knowledge the Mn of PLGA was fixed at a higher molecular weight (Mn = 50,000 Da, Mn of PAGE was kept as before at approximately 1,000 Da) and the Mn of the PEG-block was increased from 2,000 Da to 4,300 Da (PPP 3-6). The influence of the varying PEG block length was the same as observed for the PEG-*b*-PLGA: no significant increase in loading (10-11.4%) with increasing PEGylation was obtained. However, a significantly higher load of PEG-*b*-PAGE-*b*-PLGA polymers was determined compared to both PLGA alone and the PEG-

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polymers with the same degree of PEGylation. In contrast, the PEG-*b*-PAGE-*b*-PLGA polymer (PPP 7, loading 12.5 ± 0.5%) with the largest PAGE (1,500 Da) and PEG (5,550 Da) blocks provided a 35% and 30% higher loading compared to PLGA and PEG-*b*-PLGA NPs respectively. Excluding PPP 1 and PPP 2 with their lower Mn of PLGA (resulting in a significantly lower loading), an average loading for PEG-*b*-PAGE-*b*-PLGA NPs of 11.0 ± 1.8% was determined, which is overall 25% higher than the loading of the other polymers. From these loading studies, we conclude that the PEG-block in the PEGylated PLGAs has no significant influence on protein encapsulation, in contrast to the insertion of the PAGE building block which is determined to have a significant impact (Li et al., 2001; Tobío et al., 1998).

3.3. ADSORPTION AND ACTIVITY STUDIES

To evaluate the loading of various NP formulations in more detail, an adsorption and activity study with 3 representative polymers of PLGA, PEG-*b*-PLGA and PEG-*b*-PAGE-*b*-PLGA was performed. The aim of this study was to understand the influence of protein adsorption on the NP surface in contrast to protein encapsulation into the NPs, using different polymers. Additionally, the structural stability of the protein during encapsulation and after release was under investigation.

We compared the PLGA NP formulation with the highest Mn of 38,800 Da (P 5) with the PEGylated PLGA with having a PEG-block of 2,000 Da and the smallest PLGA block (PP 2). Furthermore, a PEG-*b*-PAGE-*b*-PLGA with the same PEG block Mn and the closest Mn_{PLGA} was used (PPP 3). Three additional particle batches were prepared using these polymers (Table 2). Blank and OVA-loaded NPs prepared from each polymer were found to show comparable size, PDI, zeta potential and loading. As a control purely surface-loaded NPs were also prepared in the case of each polymer, by incubation of blank NPs in OVA solution.

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For correlating the adsorption of the hydrophilic model protein OVA to the surface polarity of the particles, the surface hydrophobicity of produced NPs with surface-adsorbed OVA was determined using the hydrophobic RB dye as a reference system (Doktorovova et al., 2012; Müller et al., 1997). Plotting the PQ of RB against the surface area of the NPs should result in a straight line. The slope of each plot is then taken as a measure for the surface hydrophobicity, with a steeper the slope indicating a more hydrophobic surface (Müller et al., 1997). The RB Assay allows for a comparison of the surface properties of the different produced NPs. The larger slope for PLGA NP PQ vs. surface area profiles (P 5) indicates a relatively more hydrophobic NP surface compared to PEG-*b*-PLGA (PP 2) and PEG-*b*-PAGE-*b*-PLGA NPs (PPP 3), for which profiles with more shallow gradients were determined (Figure 4).

The amount of OVA adsorbed to the same surface-loaded NPs from the RB assay was also quantified, in order to determine the surface loading of such formulations. In accordance with the higher degree of surface hydrophobicity as determined in the RB assay, PLGA NPs showed the lowest OVA surface loading of 0.16 ± 0.01%. In contrast, surface loading of PEGylated PLGA NPs and PEG-*b*-PAGE-*b*-PLGA NPs was found to be 0.47 ± 0.04% and 0.5 ± 0.03%, respectively. The difference between the PLGA and PEGylated PLGAs polymers is of course the presence of the hydrophilic PEG-chains, which are located on the surface of the NPs. The adsorption of hydrophilic substances to such a surface is therefore reported to be better than to the more hydrophobic PLGA surface (Jeyachandran et al., 2009), in agreement with the current observations. The PAGE group seems not to impact on surface loading; this is also reflected in the RB assay, which revealed the same surface polarity for NPs prepared from PEG-*b*-PLGA and PEG-*b*-PAGE-*b*-PLGA. Although the PEGylated PLGAs show an increased protein adsorption this had no strong consequence on the comparative overall loading efficiency. Therefore it can be concluded that the higher overall loading observed for the PEG-*b*-PAGE-*b*-PLGA polymers is the result of a matrix effect of the polymer building block**.** The adsorption

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studies (Table 2) further revealed, as already described previously with respect to NP characterization (Figure 2), a load dependent decrease of the zeta potential of the NPs, regardless of whether OVA was loaded into or adsorbed onto the NPs. The zeta potential of the surface-loaded NPs lies between the zeta potential of the loaded and the unloaded NPs in the case of each polymer, which is particularly interesting with respect to the difference in zeta potential of OVA-loaded particles as compared to the surface coated NPs. From this experiment we can conclude that the lower zeta potential of the OVA-loaded NPs is not only caused by the presence of adsorbed protein. Surface loaded NPs were prepared by incubating blank NPs for only 1 h with 20 mg of OVA, which led to a surface adsorption but no encapsulation of OVA into the NPs. Compared to the surface loaded NPs, the zeta potential of the protein loaded NPs is of a lower magnitude, due not only to the protein which is obviously adsorbed at the surface, but also in some way to the encapsulated protein itself.

In addition to the adsorption measurements an ELISA was performed to check the activity of OVA following its release from NPs of the different polymers. The NP encapsulation of the protein offers a potential protection from environmental influences, preventing aggregation or cleavage and allowing for retention of biological activity (Weert et al., 2000). However, the encapsulation procedure utilizes both organic and aqueous solvents, which, in addition to the final freeze drying of the NPs, could decrease the amount of native OVA. For all NPs the amount of OVA released within 24 h was determined, and the activity of released OVA was quantified. For the surface loaded NPs an activity of approximately 40% was found, which is not unexpected, as the protein was not completely protected from degrading environmental influences. In the case of NPs where OVA was encapsulated, a higher activity of OVA was found; activity was further seen to increase in the order of PLGA (~ 60%) < PEG-*b*-PLGA (~ 65%) < PEG-*b*-PAGE-*b*-PLGA (~ 75%). This leads to the conclusion that (1) the encapsulation of proteins confers protection from negative environmental influences to a greater

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extent than surface adsorption and (2) the PEGylated PLGA polymers and, to an even greater degree, the PEG-*b*-PAGE-*b*-PLGA polymers are able to preserve the activity of encapsulated protein.

3.4. OVA RELEASE FROM THE PARTICLES

The OVA release from three batches of NPs made of the different polymer types was determined at definite time intervals up to 7 days (1, 2, 4, 8, 24, 48, 72, 144 and 168 h) in PBS buffer pH 7.4 at 37 °C. Generally, NPs made of PLGA show a biphasic release profile, with an initial burst release followed by a sustained release phase (Makadia and Siegel, 2011). The fast initial burst release is induced by the fast release of protein at or near to the surface of the NP, whereas the sustained release is caused by additional and slower release of protein from the NP polymer matrix through channels. The water inside the matrix hydrolyzes the polymer into soluble polymer products, which forms a passage for protein to be released by diffusion and erosion until complete polymer solubilization has occurred (Makadia and Siegel, 2011). The aim of determining relative protein release kinetics was to compare and evaluate the effects of the various compositions of polymer subtypes, possessing different Mn's for PEG, PAGE and PLGA as well as different lactic:glycolic acid ratios. For every PLGA polymer type respective release studies were performed, to determine the impact of both overall polymer composition as well as individual polymer building block character. Considerable differences in release behavior between the particles made from different polymers - PLGA, PEG-*b*-PLGA and PEG-*b*-PAGE-*b*-PLGA - were observed.

Different Mn of PLGA, PEG, PAGE or lactic:glycolic acid ratios within a polymer type had no major influence. However, when comparing between the polymer types, a total release of 8.8 ± 0.4% for PLGA, 11.1 ± 0.2% for PEG-*b*-PLGA and 16.9 ± 1.1% for PEG-*b*-PAGE-*b*-PLGA over 168 h was found (Figure 5A). This correlates to a 25% higher release of OVA from PEG-*b*-

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PLGA in comparison to PLGA, and a nearly 100% or 50% higher release of OVA from PEG-*b*-PAGE-*b*-PLGA compared to PLGA or PEG-*b*-PLGA. The lack of influence of the different Mn of PLGA or the lactic:glycolic acid ratio on protein release within a particular polymer group could be due to the degradation behavior of the polymers. Systematic studies of the degradation of the well-known PLGA with different lactic:glycolic acid ratios over 70 days or longer, show a faster degradation for a PLGA lactic:glycolic acid ratio of 50:50 and slower degradation for PLGA with a lactic:glycolic acid ratio of 75:25 (Lu et al., 2000, 1999). However, the difference in release between these PLGAs is typically visible only after 10 to 20 days and is almost negligible for the first days. Besides the lactic:glycolic acid ratio, the Mn of the PLGA backbone structure is important. From literature it can be concluded that with higher molecular weight slower degradation of the polymer is expected, and therefore a slower release of drugs can be hypothesized (Makadia and Siegel, 2011). This lower degradation and as a consequence slower release, is also however not visible in the first 10 to 20 days.

Looking at Figure 5A one might get the impression that the difference in release noted between NPs of the three polymer groups is mainly due to the different extent of burst release. That is not the case however, as can be clearly seen when the burst release (Figure 5B) is depicted separately from the sustained release phase (Figure 5C). For a better comparison of the sustained release curves, the difference in release was displayed compared to the 24 h value and the error bars were excluded for clarity. The burst release is explained, as mentioned before, by the fast release of the cargo located either at or near to the particle surface; this burst release is then followed by a slower, sustained release phase. The decrease in release and transition from the burst to the sustained release phase can be seen as a gradual transition in the release profile, determined to be between 8 h to 24 h for our polymers. Therefore, the release within 24 h (burst release) and the release from 24 h until 168 h (sustained release) were plotted separately. For PLGA and PEG-*b*-PLGA a lower extent of burst release of 8 to 10% was found, while a greater burst release of 14% for PEG-*b*-PAGE-*b*-PLGA was noted

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(Figure 5B). This corresponds to an increase of 4-6% in protein release from PEG-*b*-PAGE-*b*-PLGA polymer NPs in comparison to PLGA and PEG-*b*-PLGA within the first 24 h. Taking the adsorption studies into consideration, where an additional 0.3 to 0.4% of protein adsorbed to the surface of the PEGylated polymer NPs was found, the additional release of PEG-*b*-PLGA as compared to PLGA may be explained; however, this does not account for the significantly higher burst release of protein noted to occur from the PEG-*b*-PAGE-*b*-PLGA NPs. For a better comparison of the sustained release curves, the differences in release were displayed from the 24 h value onwards and the error bars excluded for clarity (Figure 5C). A slower sustained release level of 0.7% for PLGA NPs and 1.2% for PEG-*b*-PLGA NPs was observed, compared to a higher sustained release of 2.7% for PEG-*b*-PAGE-*b*-PLGA NPs.

Considering the burst and sustained release phases together, it can be seen that the PEGylation of PLGA led to an increase in the extent of protein release, which can be explained by the properties of PEG in the polymer. It is known that PLGA polymers interact with tensioactive proteins such as OVA (de Feijter, J. A., & Benjamins, 1987); this provided the possibility to form uniform NPs by a double emulsion method, but led also to the formation of compact NPs exhibiting a slow and low release of protein. By addition of another surface active molecule such as PEG, which is covalently linked to the parent polymer, the interaction between polymer and protein is reduced – this was reflected in the higher release rates observed from PEG-*b*-PLGA NPs (Blanco and Alonso, 1998). However the presence of the PEG building block cannot account for the significantly higher burst and prolonged protein release over 168 h seen for the PEG-*b*-PAGE-*b*-PLGA polymer. This could however be explained empirically by the properties of the PAGE building block in the polymer. The PAGE group adds a branched functionality to the typically linear polymer that could reduce the occurrence of steric interactions between polymer side chains. This could lead to the formation of voids or channels in the NP or a modified

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degradation behavior of the polymer, and therefore higher burst and sustained release of the protein.

In summary, PEGylation of PLGA alone had no major impact on OVA loading (PLGA NPs 8.2%; PEG-*b*-PLGA NPs 8.7%), but the addition of the PAGE building block between PEG and PLGA resulted in an increase in OVA loading (PEG-*b*-PAGE-*b*-PLGA 10.1-11%). In contrast to protein loading, the PEGylation of PLGA alone had an effect on the release of protein from formed NPs. After 168 h PEG-*b*-PLGA showed a slightly higher release of 11.1% of originally incorporated OVA, as compared to PLGA NPs which released 8.8% of incorporated protein. This difference can be attributed to the PEG building block. For PEG-*b*-PAGE-*b*-PLGA NPs the total protein released was 16.9%. i.e. nearly twice as much as from plain PLGA NPs. The different polymer building blocks were observed to influence not only the released fraction of protein, but also the release rate. An increased sustained release over 168 h could be shown for PLGA, PEG-*b*-PLGA and PEG-*b*-PAGE-*b*-PLGA, with values of 0.7%, 1.2% and 2.7% respectively. This modified release behavior can also be attributed to the different polymer compositions, rather than to different burst release profiles or adsorption effect of protein on the NP surface. Ultimately, the improved loading and release of OVA from NPs prepared from modified PLGA was seen to be accompanied by a higher stability of the OVA, as demonstrated by a higher fraction of intact and active protein. As the impact of the protein on the delivery system differs from protein to protein, the data presented in the current work cannot be directly transferred to other protein payloads. However, our evaluation of the different polymer NPs using OVA as a model has already been successfully translated to another, therapeutically relevant protein (Baleeiro et al., 2015).

4. CONCLUSION

In this work, the effect of different PEG-based modifications of PLGA on the preparation of OVA-loaded NPs was studied, with the major objective to improve protein loading and release. For

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comparison, NPs were always prepared using the same protocol, using either plain PLGA, simply PEGylated PLGA, and PEG-*b*-PAGE-*b*-PLGA. The latter modification might also allow to eventually couple some targeting moieties. Morphological characterization demonstrated a spherical shape of the NPs produced from all polymers with an average diameter of approximately 200 nm, as determined by DLS and SEM. PEG-*b*-PLGA increased the average protein load of PLGA NPs by 6%, while PEG-*b*-PAGE-*b*-PLGA increased it by 23%. The same modifications also increased the average protein release by 25% and 100%, respectively. Both effects can be clearly attributed to the different composition of the NP-forming polymers. We conclude that PEGylation of PLGA and especially the insertion of the PAGE group led to improved loading and release for the model protein OVA from such PEG-*b*-PAGE-*b*-PLGA NPs, demonstrating the potential of such a delivery system for various application routes.

5. ACKNOWLEDGEMENTS

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Figure 1

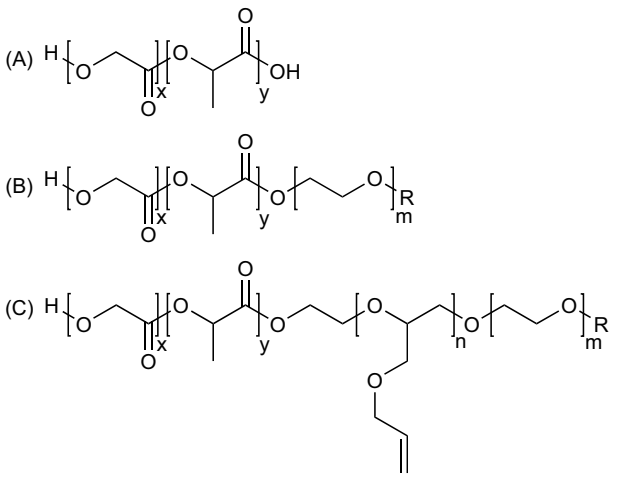

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Figure 2

Nanoparticle	Size [nm]	PDI	Zeta potential [mV]
PLGA - Blank NPs	199.7 ± 6.2	0.06 ± 0.02	-30.4 ± 1.4
PLGA - OVA loaded NPs	218.4 ± 9.7	0.11 ± 0.03	-23.8 ± 3.8
PEG-*b*-PLGA - Blank NPs	173.1 ± 7.8	0.07 ± 0.02	-29.5 ± 3.1
PEG-*b*-PLGA - OVA loaded NPs	188.4 ± 12.9	0.11 ± 0.02	-18.7 ± 2.2
PEG-*b*-PAGE-*b*-PLGA - Blank NPs	178.5 ± 11.0	0.07 ± 0.04	-27.5 ± 3.1
PEG-*b*-PAGE-*b*-PLGA - OVA loaded NPs	192.5 ± 5.3	0.11 ± 0.03	-17.7 ± 2.8

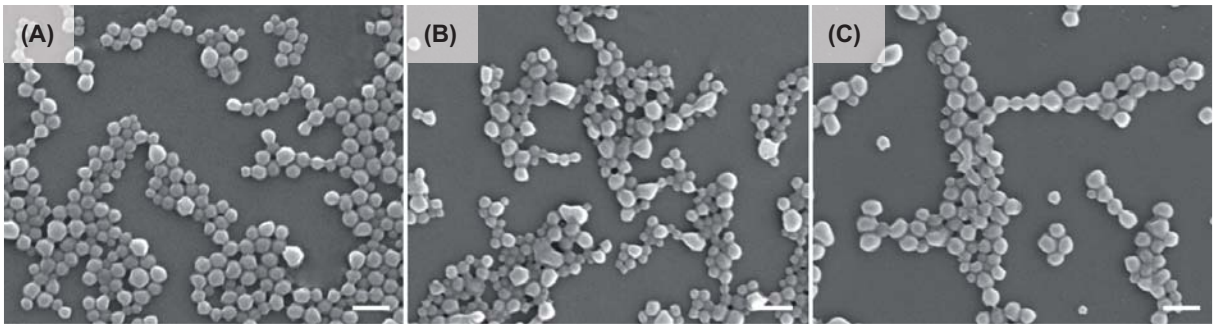

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Figure 3

Nanoparticle	PEG [Da]	PAGE [Da]	PLGA [Da]	Loading efficiency [%] [a]	Encapsulation efficiency [%] [a]
P 1	-	-	10 800	8.8 ± 1.8	44.0 ± 9.0
P 2	-	-	23 200	8.6 ± 0.5	43.0 ± 2.5
P 3	-	-	23 900	7.8 ± 0.6	39.0 ± 3.0
P 4	-	-	24 400	8.7 ± 0.3	43.5 ± 1.5
P 5	-	-	38 800	7.9 ± 0.4	39.5 ± 2.0
PP 1	1 500	-	54 500	8.5 ± 0.2	42.5 ± 1.0
PP 2	2 000	-	45 000	8.5 ± 0.9	42.5 ± 4.5
PP 3	3 200	-	46 700	9.2 ± 0.6	46.0 ± 3.0
PPP 1	2 000	850	19 150	7.7 ± 0.5	38.5 ± 2.5
PPP 2	2 000	1 000	29 700	8.2 ± 0.5	41.0 ± 2.5
PPP 3	2 000	1 000	54 250	11.4 ± 0.7	57.0 ± 3.5
PPP 4	2 400	900	54 630	10.1 ± 0.3	50.5 ± 1.5
PPP 5	2 900	1 000	49 050	10.0 ± 0.6	50.0 ± 3.0
PPP 6	4 300	1 000	51 100	11.0 ± 1.0	55.0 ± 5.0
PPP 7	5 550	1 500	44 750	12.5 ± 0.5	62.5 ± 2.5

[a] p value > 0.05 not significant (n.s), p values < 0.05 significant

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Figure 4

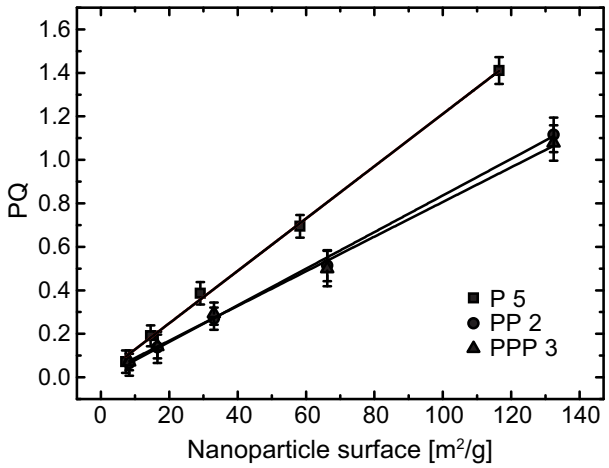

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Figure 5

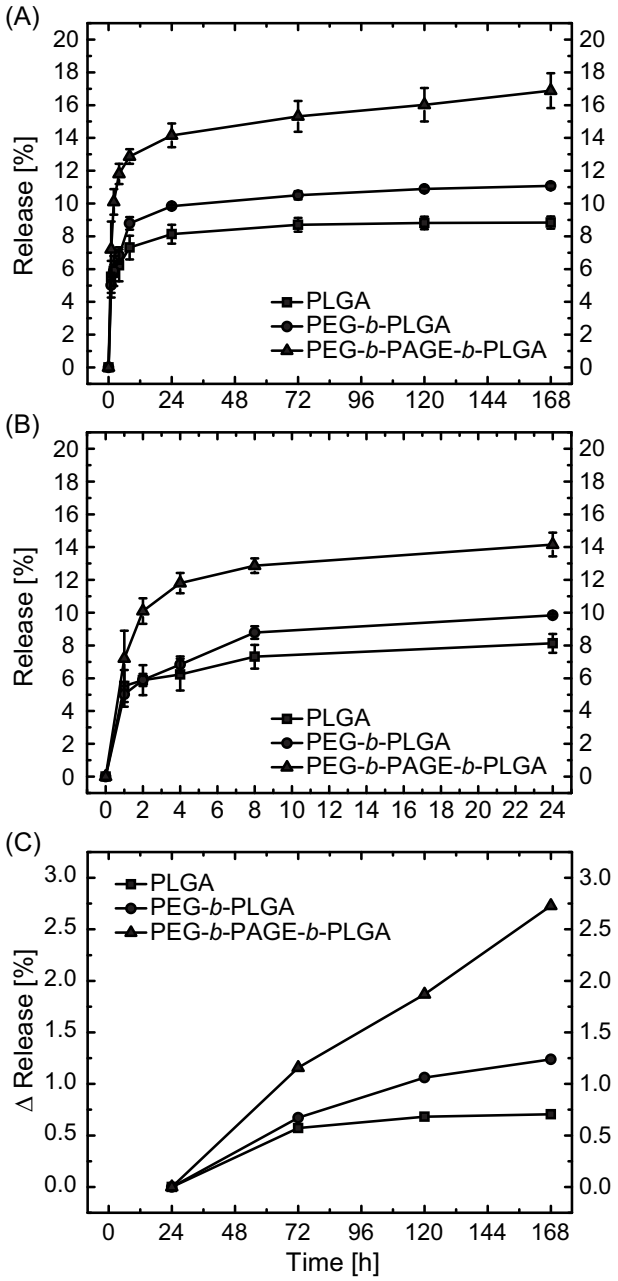

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Table 1. Composition of PLGA-Resomer types and synthesized PEG-*b*-PLGA/PEG-*b*-PAGE-*b*-PLGA polymers; PLGA as a co-polymer was considered as a monoblock.

Number	Polymer	Polymer Type	Lactic-to-glycolic acid ratio	PEG [DA]	PAGE [Da]	PLGA [Da]	Ratio (PEG: PLGA)
P 1	Resomer RG 502 H	Monoblock-	50:50	-	-	10 800	-
P 2	Resomer RG 503 H	Monoblock	50:50	-	-	23 200	-
P 3	Resomer RG 653 H	Monoblock	65:35	-	-	23 900	-
P 4	Resomer RG 753 H	Monoblock	75:25	-	-	24 400	-
P 5	Resomer RG 504 H	Monoblock	50:50	-	-	38 800	-
PP 1	PEG_{34}-*b*-$PLGA_{833}$	Diblock	50:50	1 500	-	54 500	1 : 36
PP 2	PEG_{45}-*b*-$PLGA_{694}$	Diblock	50:50	2 000	-	45 000	1 : 22.5
PP 3	PEG_{72}-*b*-$PLGA_{718}$	Diblock	50:50	3 200	-	46 700	1 : 14.5
PPP 1	PEG_{45}-*b*-$PAGE_{8}$-*b*-$PLGA_{294}$	Triblock	50:50	2 000	0 850	19 150	1 : 9.6
PPP 2	PEG_{45}-*b*-$PAGE_{9}$-*b*-$PLGA_{458}$	Triblock	50:50	2 000	1 000	29 700	1 : 15
PPP 3	PEG_{42}-*b*-$PAGE_{9}$-*b*-$PLGA_{833}$	Triblock	50:50	2 000	1 000	54 250	1 : 27
PPP 4	PEG_{54}-*b*-$PAGE_{8}$-*b*-$PLGA_{840}$	Triblock	50:50	2 400	0 900	54 630	1 : 23
PPP 5	PEG_{66}-*b*-$PAGE_{9}$-*b*-$PLGA_{754}$	Triblock	50:50	2 900	1 000	49 050	1 : 17
PPP 6	PEG_{98}-*b*-$PAGE_{9}$-*b*-$PLGA_{786}$	Triblock	50:50	4 300	1 000	51 100	1 : 12
PPP 7	PEG_{125}-*b*-$PAGE_{13}$-*b*-$PLGA_{687}$	Triblock	50:50	5 550	1 500	44 750	1 : 8

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Table 2. Results for loading and adsorption studies of PLGA (P 5), PEG-*b*-PLGA (PP 2) and PEG-*b*-PAGE-*b*-PLGA NPs (PPP 3). Data in the table represent mean ± SD (n=3).

Nanoparticle	Size [nm]	PDI	Zeta potential [mV]	Loading [%]	Activity [%]
P 5 - Blank NPs	192.3 ± 2.3	0.03 ± 0.02	-27.5 ± 1.1	0	0
P 5 - Surface loaded NPs	198.6 ± 17.3	0.10 ± 0.06	-25.9 ± 1.9	0.16 ± 0.01	38.86 ± 4.66
P 5 - OVA loaded NPs	195.0 ± 1.1	0.06 ± 0.02	-20.9 ± 1.6	7.73 ± 0.38	59.14 ± 12.31
PP 2 - Blank NPs	178.4 ± 1.9	0.05 ± 0.02	-28.8 ± 1.4	0	0
PP 2 - Surface loaded NPs	176.8 ± 1.2	0.07 ± 0.02	-23.1 ± 1.1	0.47 ± 0.04	43.5 ± 1.00
PP 2 - OVA loaded NPs	190.3 ± 1.4	0.06 ± 0.02	-18.1 ± 2.5	8.66 ± 0.87	64.89 ± 6.34
PPP 3 - Blank NPs	178.4 ± 2.3	0.06 ± 0.02	-30.9 ± 0.7	0	0
PPP 3 - Surface loaded NPs	186.5 ± 9.9	0.10 ± 0.05	-24.7 ± 2.6	0.50 ± 0.03	36.59 ± 2.82
PPP 3 - OVA loaded NPs	189.1 ± 1.8	0.08 ± 0.02	-13.0 ± 1.5	11.0 ± 0.85	74.32 ± 13.11