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# Surface Modification of PLGA Particles: The Interplay between Stabilizer, Ligand Size, and Hydrophobic Interactions

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Therapeutic and diagnostic carriers can be functionalized with active targeters to induce tissue-specific delivery. However, the possible impact of adsorbed steric stabilizer such as the frequently used poloxamers (Pluronics) on surface modification of poly(D,L-lactide-*co*-glycolide) (PLGA) particles has not been examined so far. Therefore, three model ligands of different molecular weights (653; 36 000; 155 000 g/mol) covering the size range of important targeters were conjugated to the surface of PLGA microparticles in the presence of different concentrations of Pluronic F68 (0.01−5%, w/v). Flow cytometry and fluorimetric quantification revealed for all tested ligands that high Pluronic concentrations decreased the coupling efficiency to a half or even one-third of that achieved in the absence of stabilizer. Moreover, the reduction strongly depends on the ligand size and its propensity for hydrophobic interactions. Apart from that, a high degree of particle aggregation was observed with Pluronic concentrations below 0.1% (w/v). Thus, a compromise has to be found, which combines sufficient stability with the best possible ligand coupling efficiency. For the studied system, 0.1% (w/v) turned out to be the optimum concentration of Pluronic F68.



## Introduction

Today, site-specific drug delivery is one of the most prominent challenges in biopharmaceutics. It aims at more efficient therapies and less side effects due to higher drug concentrations at the target site and fewer drug attaining healthy tissues. This is particularly important for poorly soluble hydrophobic substances, for highly active drugs such as cancer therapeutics, and for chemically instable substances including active pharmaceutical ingredients (APIs) from the biotech pipeline such as proteins, peptides, and nucleic acids.[1,2] Submicrometer or microparticulate systems might be a step forward toward improved drug delivery.[3] Additionally, they can be functionalized with certain biorecognitive ligands to specifically deliver their drug load to the diseased tissue. Numerous proof-of-concept studies have demonstrated the great potential of such strategies, but there are still huge challenges on the way to successful clinical applications. Some of these hurdles lie in the safe, efficient, and reproducible production of delivery devices, especially if grafting with active targeters is required.

The most important targeters comprise large molecules such as antibodies, e.g., anti-HER-2, and lectins, e.g., wheat germ agglutinin (WGA),[4−9] but also small molecules such as folate (MW 441 g/mol).[10] Among the potential carriers for targeted delivery, micro- and submicrometer-particles based on the biocompatible and biodegradable polymers polylactide, polyglycolide, and poly(D,L-lactide-*co*-glycolide) (PLGA) play a major role.[11,12] Various methods for PLGA particle preparation have been developed, enabling the efficient incorporation of lipophilic substances, but also the encapsulation of proteins and other hydrophilic molecules.[13,14] Most of these methods require stabilizers, e.g., for the frequently used emulsion/solvent evaporation techniques, where the surfactant prevents the coalescence of the emulsion droplets during preparation as well as the aggregation of particles during storage. At this, the most prominent stabilizers are poly(vinyl alcohol) (PVA) and poloxamers (Pluronics).[15,16] Pluronics are amphiphilic PEO−PPO−PEO triblock copolymers with hydrophilic poly(ethylene glycol) chains (PEO) linked to a more hydrophobic poly(propylene glycol) (PPO) backbone. PPO has a high affinity to the particle surface, whereas the PEO moieties are extended toward the aqueous dispersant. At low concentrations, single Pluronic molecules adsorb onto the surface and form a monolayer with the PEO chains building a "mushroom-like" conformation.[17] However, for efficient steric stabilization, a denser Pluronic adsorption layer is needed. At these higher surface concentrations, the PEO chains are more extended and build a "brush-like" conformation. The adsorbed amounts reach a plateau at bulk polymer concentrations that are still below

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the critical micelle concentration (cmc). The thickness of the adsorption layer depends on the hydrophobicity of the particle surface and the hydrophilic−lipophilic balance (HLB) of the respective Pluronic type. For Pluronic F68, a 20 nm thick layer has been measured on the surface of PLGA particles. At concentrations around or above the cmc, the layer grows even thicker due to the adsorption of hemimicelles.[18]

Another important mechanism for the stabilization of dispersions is based on the electrostatic repulsion of charged surfaces. Particles prepared from PLGA types with uncapped end groups, i.e., free carboxylate groups, have a negatively charged surface, which can be estimated by measuring the zeta potential. These carboxylate groups on the particle surface are necessary for covalent functionalization of PLGA particles with ligands. For this purpose, the carbodiimide method, which mediates the formation of an amide bond between a carboxylate and a primary amine, is the most prominent cross-linking procedure.[19,20] However, in the presence of Pluronic, the surface electrostatic potential is reduced, because part of the charged groups is shielded by the adsorbed surfactant.[18,21] Hence, we assumed that functionalization of the particle surface might be affected by the presence of adsorbed poloxamer.

Considerable effort has been put into studying the influence of stabilizers on particle size, morphology, drug incorporation, and release characteristics, as well as into the physicochemical characterization of surfactant adsorption onto polymer particles and the examination of a potential stealth effect due to strongly adsorbed hydrophilic polymers.[15,16,21,22] Concerning covalent surface functionalization, many parameters such as reagent concentrations and ratios, incubation time, and temperature have been taken into account for optimizing the coupling procedure, but the possible impact of frequently used stabilizers has not been adequately considered so far, except for PVA. For this stabilizer, McCarron et al. reported a reduction of the conjugated amount of polyclonal antibody to PLGA nanoparticles by 48% in the presence of PVA.[23]

Therefore, the aim of the present work was to determine the influence of different concentrations of the steric stabilizer Pluronic F68 on the conjugation of three fluorescent or fluorescein-labeled model ligands, namely, immunoglobulin G (IgG; MW ∼ 155 000 g/mol), WGA (MW ∼ 36 000 g/mol), and fluorescein-cadaverine (F-Cad; MW 653 g/mol), to the surface of PLGA microparticles.

## Experimental Section

**Materials.** Resomer RG502H (PLGA, 50:50 lactide/glycolide) was purchased from Boehringer Ingelheim (Ingelheim, Germany). 5-((5-Aminopentyl)thioureidyl)-fluorescein dihydrobromide (F-Cad) was obtained from Invitrogen (Carlsbad, CA). Fluorescein-labeled WGA (F-WGA, molar ratio fluorescein/protein (F/P) = 2.9) was purchased from Vector Laboratories (Burlingame, USA). Antimouse IgG-fluorescein isothiocyanate (FITC) (F-IgG, antibody developed in goat, F/P = 4.4) and Pluronic F68 were obtained from Sigma Aldrich (Vienna, Austria). All other chemicals were of analytical purity.

**Preparation and Characterization of Surfactant-Free PLGA Microparticles.** Spray-dried PLGA microparticles were prepared as previously described by Ertl et al.,[20] suspended in water and fractionated according to their size. Briefly, a solution of PLGA in methylene chloride was spray-dried with a Mini Spray Dryer B-191 (Buechi Labortechnik AG, Flawil, Switzerland). Spray-dried particles (100 mg) were suspended in 10 mL of distilled water by sonication for 5 s (Bandelin electronic UW 70/HD 70, tip: MS 72/D, Berlin, Germany) and kept for 5 min in a cooled ultrasonic bath (90% of the maximum power, Bandelin Sonorex Super 10 P, Berlin, Germany). Remaining aggregates and larger particles were spun down at 33 × *g* for 2 min. Subsequently, the supernatant was further centrifuged for 10 min at 2135 × *g*, and the pellet was resuspended in 5 mL distilled water. An aliquot was used for the quantification of the PLGA content of the resulting suspension. For scanning electron microscopy (SEM), the particles were placed on sample holders, sputter-coated with gold, and imaged in high vacuum using a Philips XL-30 ESEM (Philips Electron Optics, The Netherlands).

**Covalent Surface Modification.** In the absence and in the presence of 0.01, 0.025, 0.05, 0.1, 0.5, 1, and 5% (w/v) Pluronic F68, fluorescent ligands were coupled to the particle surface via a carbodiimide method. For this purpose, an aliquot of the microparticle suspension containing 5 mg PLGA was centrifuged for 10 min at 1300 × *g* at 4 °C. The pellet was resuspended in 500 μL 20 mM HEPES/NaOH/Pluronic pH 7.0 using a vortex mixer and an ultrasonic bath (1 min, 50% of the maximum power), and the particles were preincubated for 1 h with the respective Pluronic concentration. The suspension was centrifuged again, and the pellet was resuspended in 700 μL of a freshly prepared solution of 18 mg of *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDAC) and 0.75 mg *N*-hydroxysuccinimide (NHS) in 20 mM HEPES/NaOH/Pluronic pH 7.0. To activate the carboxylate groups on the particle surface, the suspension was incubated by end-over-end mixing at room temperature for 2 h. In order to remove excess reagents, the particles were washed twice with 20 mM HEPES/NaOH/Pluronic pH 7.4. After resuspension in the same buffer, the fluorescent ligands F-Cad (1.5 nmol, 0.98 μg), F-WGA (0.3 nmol, 10.8 μg) or F-IgG (0.03 nmol, 4.65 μg) were added. The reaction volume was adjusted to 500 μL followed by an overnight incubation. Unreacted NHS-ester groups were saturated by incubation with 10 mg of glycine dissolved in 200 μL of the same buffer for 30 min. After four washing cycles, the particles were suspended in 500 μL of 20 mM HEPES/NaOH/Pluronic pH 7.4. The amount of nonspecifically bound ligand was determined in the absence and in the presence of 1% (w/v) Pluronic following the described procedure but omitting the activation step.

**Flow Cytometry.** The particle suspensions were analyzed in terms of size distribution, surface roughness, and particle-associated fluorescence intensity using a Coulter EPICS XL-MCL Flow Cytometer (Beckman Coulter, Nyon, Switzerland). Prior to the analysis, 2−10 μL of the suspension were diluted in 1 mL 0.2% (w/v) aqueous Tween 20. The fluorescence intensity was measured at 488/525 nm. The applied forward scatter (FS) settings allowed for the analysis of particles between 1 and 10 μm as determined with polystyrene size standards. However, for data evaluation, only particles between 1 and 3 μm were considered according to the FS signal. For each analysis, 3000 particles in the respective gate were measured. Data analysis was conducted using Beckman Coulter Expo 32 Software v1.2.

**Quantification of PLGA.** The PLGA content of the particle suspension was determined by a derivatization/high-performance liquid chromatography (HPLC) protocol modified from Ding and Schwendeman.[24] In brief, a representative aliquot (200 μL) of the particle suspension was subjected to alkaline hydrolysis under harsh conditions to yield the corresponding monoacids.

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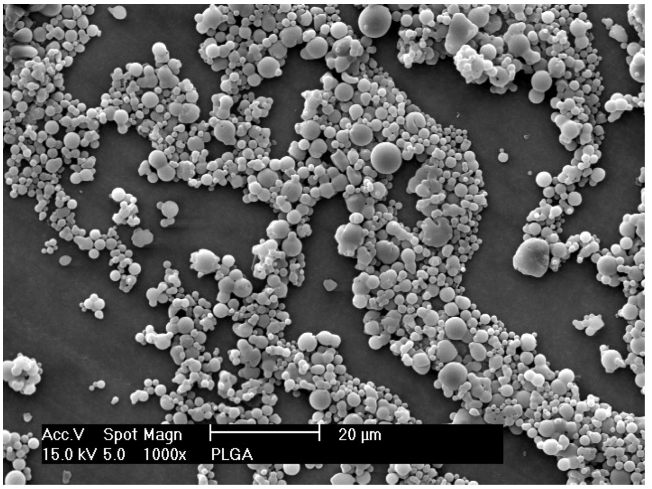


**Figure 1.** SEM of spray-dried PLGA microparticles after size-fractionation.

Subsequently, the free acids were catalytically conversed to their bromophenacyl ester derivatives in order to enhance UV absorption and improve visibility in relation to other components present in the sample mixture. Quantification was carried out on a RP-HPLC system (Agilent 1100 Series, Agilent Technologies, Santa Clara, CA) using a Lichrospher RP-18 column and gradient flow elution at a flow rate of 1.5 mL/min. Detection was done at 254 nm, and the amount of PLGA was calculated via comparison to respective standard solutions in the range of 0.01−1 mg/mL. Method validation proved excellent reproducibility, and comparative analysis showed no interference with any sample component other than PLGA.

**Quantification of Particle-Bound Ligand.** An aliquot of the particle suspension was hydrolyzed overnight in 0.1 M sodium hydroxide, and the amount of immobilized ligand in the strongly alkaline solution was determined fluorimetrically at 485/525 nm using a microplate reader (Infinite 200, Tecan Group Ltd., Grödig, Austria).

## Results and Discussion

**Treatment and Characterization of Unmodified PLGA Particles.** Surfactant-free PLGA microparticles were prepared by spray-drying and subsequent size fractionation. Because of their hydrophobicity, spray-dried particles cannot be easily suspended in surfactant-free aqueous solutions. Moreover, they contain a high amount of weakly associated aggregates. In order to improve the suspension process and to break up aggregates, ultrasonication was used. However, electron microscopy revealed that harsh sonication induced the rupture of single particles as well as melting and reaggregation due to locally increased temperatures. In order to confine the particle size distribution, aggregates that were not split up under mild conditions were removed by centrifugation. Furthermore, small particles that might be removed with the supernatant during subsequent washing steps were eliminated by an additional centrifugation step. Using flow cytometry, 68% of the unmodified particles could be gated between 1 and 3 $\mu$m. 20−25% of the remaining larger particles were found between 3 and 5 $\mu$m, which was confirmed by SEM (Figure 1). Side scatter data and SEM images revealed a smooth particle surface.

**Flow Cytometric Analysis of Particle-Associated Fluorescent Ligand.** The mean particle-associated fluorescence intensities (MFIs) of surface-modified particles gated between 1 and 3 $\mu$m were determined by flow cytometry. For better comparability, relative particle-associated fluorescence intensities (RFIs) were calculated by setting the MFI values after covalent conjugation of the respective ligand in the absence of Pluronic as 100% RFI (Table 1).

In the presence of increasing Pluronic concentrations of up to 0.5% (w/v) the amount of coupled ligands decreased strikingly. The RFI values dropped by 25% in case of the smallest ligand F-Cad and by even 40% for the large ligands F-WGA and F-IgG as compared to those without stabilizer. With 5% (w/v) Pluronic F68, the coupling efficiency of F-Cad and F-WGA was halved, and for F-IgG it even dropped to one-third.

To estimate the contribution of noncovalent binding, particles were incubated with the fluorescent ligands but omitting EDAC/NHS activation. In the absence of Pluronic, about 3% of F-WGA, and about 24% of the totally immobilized F-Cad and F-IgG were bound nonspecifically. In the presence of a high Pluronic concentration (1% (w/v)), the obtained results for nonspecific binding were similar in the case of F-Cad and F-WGA, whereas noncovalent binding was reduced to one-third in the case of F-IgG.

Thus, the major amount of particle-associated ligand was immobilized covalently, and the observed decrease in the presence of increasing Pluronic concentrations can be mainly attributed to a concurrently hindered access due to adsorbed surfactant. As initially supposed, part of the observed differences between the three tested ligands might be due to the ligand size. Steric hindrance is expected to be more pronounced for larger ligands, whereas a small molecule should rather be able to penetrate the adsorption layer. Nevertheless, size is not the only parameter. The propensity of the ligands for hydrophobic interactions also influences the surface binding as exemplified by the discrepancy between F-IgG and F-WGA. IgG is a prominent opsonin, which exerts a high degree of hydrophobic interactions.[25] Thus, for IgG, a high degree of nonspecific adsorption to the hydrophobic particle surface could be observed in the absence of Pluronic. In the presence of 1% (w/v) Pluronic, however, these nonspecific interactions are reduced as a result of increased hydrophilicity of the particle surface. This is consistent with the proposed stealth effect of adsorbed Pluronic, which should prevent the opsonization of drug carriers with complement factors and antibodies.[23] By contrast, there is only a very low extent of nonspecific binding of WGA, which is not influenced by the presence of Pluronic. Its remarkably lower affinity to the particles might result from a rather rigid and (because of disulfide bridges) highly conserved molecular structure, which counteracts hydrophobic interactions.[26] Concerning the reduced covalent coupling efficiency in the presence of Pluronic, it should be noted that steric hindrance could not only be caused by surfactant adsorption at the hydrophobic particle surface, but also by adsorption at hydrophobic regions of large protein molecules such as IgG. Pluronic might therefore block the accessibility of PLGA carboxylates on the one hand, and that of IgG amine groups on the other hand.

For the third ligand F-Cad, a high proportion of nonspecific binding in the absence of Pluronic was observed, which might be attributed to its rather hydrophobic structure. Interestingly, the RFI values in the presence of 1% (w/v) Pluronic were in a similar range. Probably part of the nonspecifically bound F-Cad was not located directly at the PLGA surface but incorporated in adhered hemimicelles. This would account for the discrepancy between the

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**Table 1. RFI of PLGA Particles (1−3 $\mu$m) after Surface Modification with F-Cad, F-WGA, and F-IgG Grafted in the Presence and in the Absence of Pluronic As Determined by Flow Cytometry (mean ± SD, $n = 5$)[a]**

| | | F-Cad | | F-WGA | | F-IgG | |
|---|---|---|---|---|---|---|---|
| MW (g/mol) | Pluronic | 653 | | 36 000 | | 155 000 | |
| | % w/v | RFI | % gated particles (1 - 3 $\mu$m) | RFI | % gated particles (1 - 3 $\mu$m) | RFI | % gated particles (1 - 3 $\mu$m) |
| total binding (covalent modification) | 0 | 100.00 ± 13.26 | 28.89 ± 3.39 | 100.00 ± 4.74 | 26.30 ± 1.89 | 100.00 ± 8.71 | 38.14 ± 1.65 |
| | 0.01 | 97.54 ± 23.80 | 28.17 ± 4.73 | 89.92 ± 4.34 | 28.26 ± 1.44 | 104.61 ± 4.07 | 33.84 ± 1.67 |
| | 0.025 | 88.02 ± 1.92 | 30.03 ± 2.88 | 73.14 ± 8.27 | 32.07 ± 3.22 | 99.98 ± 9.10 | 34.57 ± 0.94 |
| | 0.05 | 106.87 ± 13.72 | 26.23 ± 4.65 | 75.22 ± 0.80 | 40.13 ± 1.20 | 89.99 ± 5.46 | 41.81 ± 4.49 |
| | 0.1 | 95.98 ± 5.22 | 29.46 ± 3.30 | 86.18 ± 10.17 | 42.21 ± 4.64 | 87.17 ± 2.28 | 40.16 ± 1.04 |
| | 0.5 | 74.74 ± 4.78 | 29.74 ± 2.12 | 62.88 ± 2.98 | 48.81 ± 1.04 | 60.48 ± 3.16 | 51.12 ± 4.97 |
| | 1 | 64.50 ± 6.68 | 37.24 ± 2.35 | 67.42 ± 1.88 | 49.93 ± 2.50 | 48.72 ± 7.65 | 47.52 ± 5.57 |
| | 5 | 50.77 ± 5.44 | 43.76 ± 1.68 | 52.53 ± 2.44 | 48.79 ± 2.37 | 34.78 ± 2.89 | 49.29 ± 1.79 |
| noncovalent binding | 0 | 23.51 ± 1.36 | 51.48 ± 2.63 | 3.36 ± 0.14 | 51.94 ± 0.46 | 23.95 ± 2.59 | 60.59 ± 0.77 |
| | 1 | 22.86 ± 1.00 | 56.52 ± 0.57 | 3.36 ± 0.68 | 52.88 ± 0.56 | 8.39 ± 0.52 | 63.47 ± 0.97 |

[a] Percentage of particles gated (1−3 $\mu$m).

**Table 2. Ligand/PLGA Ratio after Covalent Coupling of F-Cad, F-WGA, and F-IgG to PLGA Microbeads in the Presence of Increasing Concentrations of Pluronic F68 As Determined by Fluorimetry and HPLC (mean ± SD, $n = 5$), Expressed As % Related to Covalent Coupling in the Absence of Stabilizer**

| | Pluronic % w/v | F-Cad/ PLGA | F-WGA/ PLGA | F-IgG/PLGA |
|---|---|---|---|---|
| total binding (covalent modification) | 0 | 100.00 ± 1.14 | 100.00 ± 15.10 | 100.00 ± 17.02 |
| | 0.01 | 82.95 ± 7.95 | 84.09 ± 14.62 | 96.83 ± 28.64 |
| | 0.025 | 76.14 ± 2.27 | 61.68 ± 5.70 | 98.57 ± 19.72 |
| | 0.05 | 78.41 ± 4.55 | 91.93 ± 10.16 | 95.50 ± 20.17 |
| | 0.1 | 75.00 ± 1.14 | 83.57 ± 17.47 | 96.87 ± 23.14 |
| | 0.5 | 79.55 ± 2.27 | 31.77 ± 8.07 | 64.70 ± 17.99 |
| | 1 | 71.59 ± 3.41 | 36.32 ± 5.27 | 58.40 ± 6.95 |
| | 5 | 61.36 ± 3.41 | 30.39 ± 10.73 | 29.30 ± 5.80 |
| noncovalent binding | 0 | 69.32 ± 9.09 | 5.03 ± 0.85 | 13.04 ± 2.43 |
| | 1 | 50.01 ± 2.27 | 4.51 ± 0.43 | 10.14 ± 1.68 |

impaired covalent coupling of F-Cad and the high extent of nonspecific binding co-occurring.

**Quantification of Immobilized Ligand.** In order to cover the whole range of particle sizes, the absolute amount of ligand in the suspension was determined fluorimetrically. To consider a potential loss of particles during the conjugation procedure, the PLGA content of the modified particle suspension was also quantified, and the ratio between ligand and PLGA concentration was calculated (Table 2).

As already detected by flow cytometry, the amount of coupled ligand strongly decreased with rising Pluronic concentrations. In the presence of 5% (w/v) Pluronic, the coupling rate of F-Cad decreased by 40% as compared with conjugation in the absence of stabilizer. For F-WGA and F-IgG, the values declined by even 70%. The sharpest reduction was again observed at Pluronic concentrations between 0 and 0.5% (w/v). Thus, the results obtained via flow cytometry could be confirmed.

However, the contribution of noncovalent binding is much higher than that assessed via flow cytometry, especially in the case of F-Cad. In the absence of Pluronic, 69% of the F-Cad immobilized upon covalent modification was nonspecifically associated with the particles. By contrast, the flow cytometric analysis revealed 24% nonspecific binding. This discrepancy might be explained by the additional purification step during flow cytometry, where the particles are diluted with a sheath fluid and run through capillaries with a high speed. That way, some of the noncovalently bound ligand molecules might have an affinity high enough to resist repeated centrifugation and resuspension, but too low to withstand the capillary flow and might therefore be removed before passing the detector. This effect seems to be most pronounced for F-Cad with its high affinity to hydrophobic structures. For F-IgG, despite its susceptibility to hydrophobic interactions, this effect was not noticeable. This might be due to the fact that the shear stress, which acts on larger molecules during the washing steps, is proportionally higher and thus the applied protocol results in sufficient purification. In the case of F-WGA, absolute quantification revealed about 5% of nonspecific binding, which was comparable to the data obtained by flow cytometry. This effect might be attributed to the high conformational stability of WGA and the therefore low propensity for hydrophobic interactions.[26]

**Stabilization of the Particle Suspension.** During the coupling process, one of the most important aspects is the stabilization of the particle suspension. Insufficient electrostatic repulsion and a rather hydrophobic particle surface are known to provoke particle aggregation. In addition, the quantification of the PLGA content of the suspensions after surface-modification revealed a relevant loss of particles during the coupling and washing procedures (Figure 2), which was even visible since particles were sticking to the hydrophobic surfaces of pipet tips and plastics labware. As the mechanisms that induced particle loss in our setup seem to also impair the stability of a suspension, they merit further consideration.

No relevant loss of particles was only observed for batches treated with 0.5 and 1% (w/v) Pluronic F68. Stabilizer concentrations of 0.025% (w/v) and below resulted in a substantially

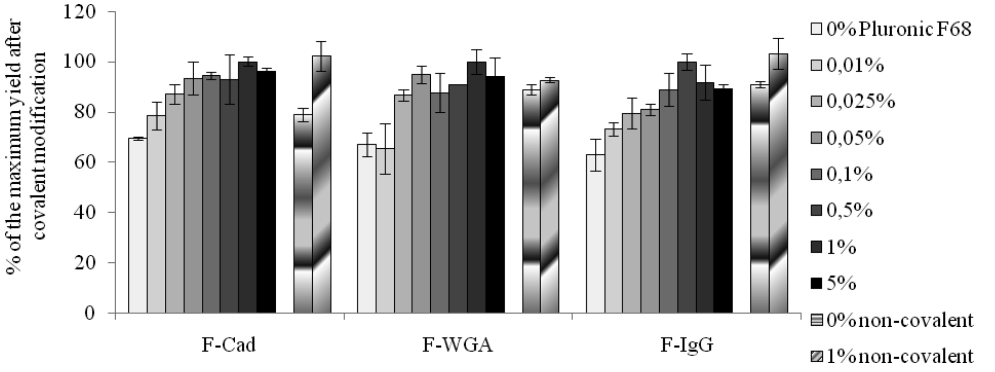


**Figure 2.** Particle yield after covalent and noncovalent modification in the presence and absence of Pluronic F68; expressed as % of the maximum yield after covalent modification.

lower yield amounting to 84% in the presence of 0.025% (w/v) poloxamer and 73% in the presence of 0.01% (w/v) surfactant at the mean. In the absence of Pluronic, particle recovery was further reduced to 63%. However, very high amounts of stabilizer (5% (w/v)) also resulted in a decline of 7%. This effect was even aggravated after carbodiimide activation. In the absence of Pluronic, the particle yield after covalent modification was 12% (F-Cad), 24% (F-WGA), or 31% (F-IgG) lower than that without activation, while in the presence of 1% (w/v) Pluronic, EDAC/NHS treatment did not affect the particle yield. This might be attributed to the derivatization of the carboxylate group to an active ester with reduced surface potential and thus increased hydrophobicity, which impairs electrostatic stabilization.[23] The problem might be overcome by the presence of Pluronic, which renders the particle surface more hydrophilic. As a steric stabilizer Pluronic acts via osmotic effects and polymer coil compression. On the one hand, the high polymer concentration in the overlap zone induces the flow of solvent into the interparticle region, and, on the other hand, the elastic compression of polymer molecules that results in a net loss in configurational entropy pushes the particles apart.[18]

However, absolute quantification cannot reveal the proportion of aggregated particles within the suspension. As a result, the forward and side scatter signals obtained by flow cytometry offered further information (Table 1) indicating a shift of the size distribution to larger sizes and to higher granularity upon modification in the presence of low Pluronic concentrations (below 0.1% (w/v)), which points to particle aggregation. In the absence of Pluronic, covalent modification resulted in a reduction of the particle fraction gated between 1 and 3 $\mu$m by about one-third as compared to samples without carbodiimide activation, while the decrease was less pronounced in the presence of 1% (w/v) Pluronic. Thus it can be concluded that the impaired electrostatic stability was partly compensated by steric stabilization with Pluronic. While 68% of all detected particles had been between 1 and 3 $\mu$m before modification, only one-third of all particles detected were still in that size range after covalent modification in the presence of Pluronic concentrations of up to 0.1% (w/v). For higher concentrations, the number of gated particles increased to about 50%.

These observations lead back to the initial considerations. A steric stabilizer is usually not only required for successful particle preparation, but also for stabilization of suspensions during modification and storage. PLGA particle suspensions prepared by solvent evaporation technique often contain rather high Pluronic concentrations of about 1 to 2.5% (w/v).[16,27] Most of it can be removed during purification by centrifugation, diafiltration, or dialysis. However, a certain concentration threshold should usually not be under-run in order to provide sufficient steric stabilization.

## Conclusion

The present study highlights an essential topic for the design of targeted drug carriers. The obtained data clearly indicates that Pluronic F68 impairs covalent functionalization of the surface of PLGA particles in a concentration-dependent manner. As determined by flow cytometry, the coupling efficiency of F-Cad and F-WGA in the presence of high Pluronic concentrations (5% (w/v)) amounted to only about 50% of that achieved in the absence of steric stabilizer, and coupling of F-IgG representing the largest ligand tested even decreased to 35%. The absolute quantification of the amounts of coupled ligand related to the remaining PLGA revealed a similar decrease. Thus, it might be concluded that the Pluronic adsorption layer on the particle surface sterically hinders the chemical reaction between the ligand and the surficial PLGA carboxylate groups. Furthermore, ligand characteristics such as size and hydrophobicity play an important role. Therefore, future studies aiming at the functionalization of PLGA particles with active targeters should consider the amount of steric stabilizer. On the basis of the obtained results, Pluronic concentrations above 0.1% (w/v) should be avoided during covalent surface modification. A stabilizer concentration of 0.1% (w/v) might be a good compromise between sufficient stabilization and the optimum coupling efficiency. These findings might contribute to the advancement of targeted delivery systems from successful proof-of-concept studies to efficient applications.

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