Covalently Binding of Bovine Serum Albumin to Unsaturated Poly(Globalide-Co-ε-Caprolactone) Nanoparticles by Thiol-Ene Reactions

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When nanoparticles (NPs) are introduced to a biological fluid, different proteins (and other biomolecules) rapidly get adsorbed onto their surface, forming a protein corona capable of giving to the NPs a new “identity” and determine their biological fate. Protein–nanoparticle conjugation can be used in order to promote specific interactions between living systems and nanocarriers. Non-covalent conjugates are less stable and more susceptible to desorption in biological media, which makes the development of engineered nanoparticle surfaces by covalent attachment an interesting topic. In this work, the surface of poly(globalide-co-ε-caprolactone) (PGlCL) nanoparticles containing double bonds in the main polymer chain is covalently functionalized with bovine serum albumin (BSA) by thiol-ene chemistry, producing conjugates which are resistant to dissociation. The successful formation of the covalent conjugates is confirmed by flow cytometry (FC) and fluorescence correlation spectroscopy (FCS). Transmission electron microscopy (TEM) allows the visualization of the conjugate formation, and the presence of a protein layer surrounding the NPs can be observed. After conjugation with BSA, NPs present reduced cell uptake by HeLa and macrophage RAW264.7 cells, in comparison to uncoated NP. These results demonstrate that it is possible to produce stable conjugates by covalently binding BSA to PGlCL NP through thiol-ene reaction.

1. Introduction

The application of nanomaterials has attracted the attention of many research fields in the last decades. In the biomedical field, there is a special interest regarding the use of nanomaterials due to their capability to interact with cells and reach difficult access targets. The scientific and medical community has now recognized that when nanoparticles (NPs) are exposed to a biological environment, their surface is modified by the adsorption of proteins, forming the so-called protein corona. Nowadays, it is well-accepted that the presence of the protein corona affects cellular responses to the NPs, determining their biological fate. In this context, engineering the surface of the NPs is an excellent way to tune their interfacial properties and create a wide material platform for specific biological and biomedical applications.

Nanoparticles with tailored surface properties are potentially useful in a broad range of applications, promoting specific interactions between nanoparticles and biological systems. The non-covalent adsorption method is one of the most frequently employed procedures for the attachment of proteins onto particles. This is the simplest way to produce NP–protein conjugates, and the attachment occurs by hydrophobic or electrostatic interactions between the NP and the protein. However, conjugates produced by non-covalent methods are reversible, which means that proteins can adsorb and desorb from the surface of NPs, making it difficult to maintain their stability, uniformity, and reproducibility. Under physiological conditions, proteins adsorbed to the NPs can desorb and be replaced by other proteins present in the local environment, and a long-term behavior of these conjugates may be difficult to predict. In order to avoid these problems, covalent attachment of proteins to NPs becomes an alternative to produce conjugates that are stable toward dissociation. The stability and irreversibility of covalent protein–NP conjugates can be decisive factors governing the biological response of cells and organisms, making
this strategy useful for applications in complex biological media with other interfering species.[7,15]

Click reactions are an excellent approach to the preparation of biologically active protein–polymer NPs. Thiol-ene reactions are an example of a type of click reaction, consisting of a simple and adaptable methodology to prepare functionalized polymers using combinations of multi-functional alkenes and thiols.[16,17] Thiol-ene coupling present high yields, fast reaction rates, and form harmless byproducts.[18,19] These reactions are frequently employed as a post-polymerization modification in unsaturated polymers, enabling the formation of biofunctional materials.[17,20–22] Thiol-ene reactions are especially interesting in the presence of free thiol groups in cysteine-containing proteins, since this allows a click reaction with accessible groups on NPs surface, leading to site-selective and irreversible conjugations.[9]

In the present study, we aimed to produce poly(globalide-co-ε-caprolactone) (PGlCL) nanoparticles and covalently conjugate them with bovine serum albumin (BSA) protein, in order to obtain irreversible and stable conjugates, suitable for biomedical applications. PGlCL is a biocompatible unsaturated copolyester, whose properties can be tailored by tuning its monomer ratios and/or by the functionalization of its double bonds. As reported in previous studies,[23,24] PGlCL has a great potential to be used as the basis for a new biomaterials platform. The synthesis of PGlCL was carried out by enzymatic ring-opening polymerization (e-ROP) using supercritical carbon dioxide (scCO2) as solvent, and then PGlCL NPs were produced by the solvent evaporation method. The double bonds present on the surface of PGlCL NPs were then directly functionalized by thiol-ene reactions. At first, the nanoparticles were functionalized with a small molecule containing a thiol group, N-acetylcysteine (NAC), in order to give us information about the feasibility of the surface functionalization. Then, BSA was modified to increase the amount of thiol units in its structure, and it was conjugated to PGlCL NPs (Figure 1). The success of the covalent conjugation of BSA to PGlCL NPs was evaluated through flow cytometry (FC) and fluorescence correlation spectroscopy (FCS) measurements. The conjugate visualization was possible through transmission electron microscopy (TEM). Finally, cellular uptake assays were performed, comparing BSA-NPs conjugates and uncoated NPs. To the best of our knowledge these are the first studies on covalent conjugation of PGlCL NPs with BSA. It is important to highlight that this is a novel and simple strategy that can be also used for surface modifications of PGlCL NPs with other types of protein. We see our experiments as an important contribution for the development of engineered nanomaterial surfaces and for safer and more effective medical treatments.

2. Experimental Section

2.1. Materials

Solvents were purchased from Merck and Sigma Aldrich and used as received, unless otherwise stated. The thermal initiator potassium persulfate (KPS) was purchased from Thermo Fisher Scientific and the surfactant sodium dodecyl sulfate (SDS) was purchased from Alpha Aesar. The hydrosoluble photoinitiator Irgacure 2959® was purchased from BASF.

Carbon dioxide used as solvent was purchased from White Martins S/A. ε-caprolactone (CL), N-acetylcysteine (NAC), bovine serum albumin (BSA), and bovine serum albumin-fluorescein isothiocyanate conjugate (BSA-FITC) were...
the modified BSA was carried out through thiol-ene reactions, directly on the polymer chain double bonds present on the surface of the particle. The purified miniemulsion containing PGICL NPs was placed in a flask together with modified BSA and the water-soluble initiator Irgacure 2959® under nitrogen atmosphere. During the reactions, the flask was exposed to UV light (365 nm) for 4 h, under continuous magnetic stirring. The amount of BSA used was established as 10⁻⁸ mol of BSA per mL of miniemulsion. This amount was calculated to be sufficient to cover the surface of all nanoparticles. Irgacure 2959® content was fixed in 1% (mol), relative to the total amount of thiol groups from BSA present in the reaction media.

After the reaction, the miniemulsion containing covalent BSA-NPs conjugates were stored in refrigerator (4 °C), until purification was carried out. The conjugates were purified by performing centrifugation/washing cycles. Centrifugation was carried out in Amicon filtration tubes (MWCO: 100 kDa, 9000 rpm, 10 min) for the removal of free/weakly adsorbed BSA. The filtrate containing free BSA was removed and the covalent conjugates were re-dispersed in distilled water. Purified covalent conjugates were stored in refrigerator (4 °C).

Non-Covalent Functionalization of the Nanoparticles with Modified BSA: The non-covalent conjugation of PGICL nanoparticles with the modified BSA was carried out through thiol-ene reactions, without any initiator. The mixture was incubated overnight in refrigerator. The amount of BSA used was 10⁻⁸ mol of BSA per mL of miniemulsion.

2.4.2. Functionalization with Bovine Serum Albumin (BSA)

BSA Modification: Before conjugation with nanoparticles, BSA was modified by reaction with Traut's reagent (2-Iminothiolane), aiming to introduce more thiol groups to the BSA structure, which should enhance its capacity of conjugation with PGICL NPs through thiol-ene reaction. A BSA solution was prepared (10 mg BSA mL⁻¹) in sodium phosphate buffer 0.1 M, pH 8.0 containing 1 mM EDTA. The 2-Iminothiolane solution was prepared in a 2 mg mL⁻¹ concentration, and was mixed to the BSA solution in a proportion of 1:50 (BSA:2-Iminothiolane) (v/v). The reaction was carried out for 1 h in a shaker, at room temperature. After reaction, the modified BSA was purified by dialysis for 48 h (MWCO: 33 kDa) and then stored in a freezer (−18 °C).

Covalent Functionalization of the Nanoparticles with Modified BSA: The covalent conjugation of PGICL nanoparticles with the modified BSA was carried out through thiol-ene reactions, directly in the polymeric chain double bonds present on the surface of the particle. The purified miniemulsion containing PGICL NPs was placed in a flask together with modified BSA and the water-soluble initiator Irgacure 2959®, under nitrogen atmosphere. During the reactions, the flask was exposed to UV light (365 nm) for 4 h, under continuous magnetic stirring. The amount of BSA used was established as 10⁻⁸ mol of BSA per mL of miniemulsion. This amount was calculated to be sufficient to cover the surface of all nanoparticles. Irgacure 2959® content was fixed in 1% (mol), relative to the total amount of thiol groups from BSA present in the reaction media.

After the reaction, the miniemulsion containing covalent BSA-NPs conjugates were stored in refrigerator (4 °C), until purification was carried out. The conjugates were purified by performing centrifugation/washing cycles. Centrifugation was carried out in Amicon filtration tubes (MWCO: 100 kDa, 9000 rpm, 10 min) for the removal of free/weakly adsorbed BSA. The filtrate containing free BSA was removed and the covalent conjugates were re-dispersed in distilled water. Purified covalent conjugates were stored in refrigerator (4 °C).
After the incubation period, the miniemulsion containing non-covalent BSA-NPs conjugates were purified by performing centrifugation/washing cycles. Centrifugation was carried out in Amicon filtration tubes (MWCO: 100 kDa, 9000 rpm, 10 min) for the removal of free/weakly adsorbed BSA. The filtrate containing free BSA was removed and the non-covalent conjugates were re-dispersed in distilled water. Purified non-covalent conjugates were stored in refrigerator (4 °C).

2.5. Proton Nuclear Magnetic Resonance (1H NMR)

1H NMR spectroscopy was performed on a Bruker Avance 300, operating at 300 MHz. All spectra were referenced internally to residual proton signals of the deuterated solvent. Samples were solubilized in CDCl3 (δ = 7.27 ppm for 1H NMR).

2.6. Ellman’s Assay

The Ellman’s assay was used to determine the final concentration of thiol groups present on modified BSA, as well as the amount of NAC non-reacted to the NPs. Ellman’s reagent (5,5'-dithio-bis-[2-nitrobenzoic acid], DTNB) was solubilized in sodium phosphate buffer (0.1 M, pH 8.0, containing EDTA 1 mM), in a concentration of 4 mg mL⁻¹. The assay was carried out in triplicate, in a 96 well plate, mixing in each well 4 µL of Ellman’s reagent, 200 µL of buffer and 20 µL of sample. The reaction occurs under constant stirring and protected from light, during 15 min. N-acetylcysteine (NAC) was used as a standard. Absorbance was measured at 412 nm with a Tecan infinite plate reader.

2.7. Pierce Assay

The protein concentration was determined by Pierce 660 nm Protein Assay according instructions of the manufacturers. Bovine serum albumin (BSA) was used as a standard. Absorbance was measured with a Tecan infinite plate reader.

2.8. Dynamic Light Scattering (DLS)

Intensity particle average diameters of the uncoated nanoparticles and the conjugates (Dp) and the polydispersity indexes (PDI) were measured by dynamic light scattering (DLS—Malvern Instruments, Zetasizer Nano S). The latex samples were diluted approximately 1:15 with distilled water prior to DLS measurements.

2.9. Zeta Potential

The zeta (ζ) potential of the uncoated nanoparticles and the conjugates (10 µL, 10 mg mL⁻¹) was measured in a 1 mM potassium chloride solution (1 mL) with a Zeta Sizer Nano Series (Malvern Instruments).

2.10. Transmission Electron Microscopy (TEM)

Transmission electron microscopy was carried out with a FEI Tecnai F20 transmission electron microscope operated at an acceleration voltage of 200 kV. The NPs were first diluted with 1 mL water and then one droplet was placed on a carbon-coated copper grid and dried overnight.

In order to visualize the presence of the protein coating on the conjugates, a staining technique was used. NPs (uncoated and conjugates) were first diluted with water and then placed onto a lacy grid and let to dry. The samples were stained with 4% uranyl acetate + 1% trehalose solution (1:1 v/v) according to the method from Kokkinopoulos et al.[26,27] Images were taken with an Ultrascan 1000 (Gatan) charge-coupled device (CCD) camera.

2.11. Flow Cytometry (FC)

Flow cytometry measurements were performed for NPs covalently and non-covalently conjugated with BSA, and also for uncoated NPs. For these measurements, the conjugates were produced using fluorescein isothiocyanate (FITC) labeled BSA. BSA-FITC modification, covalent, and non-covalent conjugation to the NPs and purification of the conjugates were carried out as described in item 2.4.2. Removal of unbound or weakly adsorbed BSA-FITC and free FITC was monitored by fluorescence measurements and no fluorescence signal was detected in the filtrate after centrifugation/washing cycles in Amicon filtration tubes.

Flow cytometry measurements were performed using an Attune NxT Flow Cytometer (laser: 488 nm laser for FITC excitation; emission: 530 nm band pass filter). The fluorescent signal was expressed in a histogram and the amount fluorescent positive nanoparticles (%) was determined. Control measurements were performed with pure PBS. Attune NxT software was used for data analysis.

2.12. Fluorescence Correlation Spectroscopy (FCS)

FCS measurements were performed for NPs covalently and non-covalently conjugated with BSA. For this measurements, the conjugates were produced using fluorescein isothiocyanate (FITC) labeled BSA. BSA-FITC modification, covalent, and non-covalent conjugation to the NPs, and purification of the conjugates were carried out as described in item 2.4.2. The measurements were also performed for pure modified BSA-FITC.

Removal of unbound (or weakly adsorbed) BSA-FITC and free FITC was monitored by fluorescence measurements and no fluorescence signal was detected in the filtrate after centrifugation/washing cycles in Amicon filtration tubes.

FCS was carried out on a commercial setup (Carl Zeiss, Germany) consisting of the modules LSM510, ConfoCor 2, and an inverted microscope model Axiovert 200 with a C-Apochromat 40 ×, NA 1.2 water immersion objective. An argon ion laser (488 nm) was used for excitation and the emission was detected after filtering with a BP505-550 band pass.
filter. 8-Well, polystyrene chambered cover glasses (Laboratory-Tek, Nalge Nunc International) were used as sample cells. For each sample series 10 measurements with a total duration of 300 s were performed. The confocal observation volume was calibrated using a reference dye with a known diffusion coefficient in water, that is, Alexa Fluor488. The diffusion coefficients and hydrodynamic radii of the BSA-FITC and the NPs conjugates were obtained by fitting the experimental autocorrelation curves with analytical model functionassuming either one or two types of fluorescent species.[28]

2.13. Cell Culture

Hela cells were obtained from ATCC (American Type Culture Collection). The cells were maintained in Dulbecco’s modified eagle medium (DMEM) supplemented with 10% FBS, 100 U mL⁻¹ penicillin, 100 mg mL⁻¹ streptomycin and 2 mM glutamine (Thermo Fisher).

2.14. Cell Uptake via Flow Cytometry Analysis

HeLa cells (100000 cells per well) were seeded out in 24 well plates (200 μL) for flow cytometry analysis. After overnight incubation at 37 °C, cells were washed and serum-free culture medium was added. It was also tested the addition of cell culture medium containing 10% human serum or 10% fetal bovine serum (FBS). For nanoparticles uptake analysis, the fluorescent dye Coumarin-6 was first encapsulated in the NPs, and then the BSA-NPs covalent conjugates were produced. Uncoated nanoparticles and conjugates were applied to cells at a concentration of 37.5 μg mL⁻¹ for 2 h.

Afterwards, cells were collected and detached with 2.5% trypsin from cell culture wells. Flow cytometry measurements were performed with Attune NXT Flow Cytometer (Invitrogen, USA). The fluorescent dye Coumarin-6 was excited with a 488 nm laser. Data analysis was performed using Attune NXT software (Invitrogen, USA). Values are expressed as percentage (%) of fluorescent positive cells as an average of at least four independent experiments.

2.15. Statistical Analysis

Data was analyzed with GraphPad Prism 8.1.1 using a two-way Anova (Sidak’s multiple comparison test). Calculated p-values were defined as followed: *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001. Not significant differences are labelled as ns.

3. Results and Discussion

3.1. PGICL Nanoparticle Formation and Functionalization with NAC

The formation of PGICL NPs was carried out through the solvent evaporation method. At first, PGICL was synthesized by e-ROP using scCO₂ as solvent, and then pre-synthesized PGICL was used to prepare the NPs. In order to evaluate size, stability, and morphology of the NPs, DLS and zeta potential measurements were performed, as well as TEM imaging (Figure 2A). The mean particle diameter was determined to be around 146 ± 2 nm, with a low PDI value (<0.1), indicating a narrow particle size distribution. In addition, the zeta potential value was −54.1 ± 0.3 mV, indicating high colloidal stability. The negative charge is associated to the presence of SDS (an anionic surfactant) adsorbed on the surface of the particles.[29] TEM images corroborate with results obtained by DLS for average particle size and confirm the spherical morphology of the NPs.

After the NPs preparation, the double bonds present on its surface were functionalized through thiol-ene reaction with NAC, as can be seen in Figure 2B. The consumption of the double bonds in the polymer chains was determined through ¹H-NMR spectroscopy measurements, by comparing the integral values of the double bond peaks before and after functionalization with NAC. Besides, the double bond consumption caused specifically by thiol (SH) groups attachment could be determined. After the functionalization reaction, the samples were purified and the supernatant containing excess of NAC was collected. The concentration of non-reacted NAC was determined by the Ellman’s assay. Figure 2C compares the global double bond consumption and the double bond consumption caused by SH attachment. It is possible to observe that the higher the amount of initiator used, the higher is the global double bond consumption, reaching around 9% for the highest initiator concentration tested. As described in 2.4.2, NAC was used in sufficient amount to consume 5% of the double bonds, which indicate that the double bond consumption is not happening only due to the NAC coupling. On the other hand, the double bond consumption caused by SH attachment remained almost constant at a value around 4%, which is consistent to the amount of NAC used. The consumption of the double bonds may happen not only due to the SH attachment by thiol-ene reaction, but also because of other side reactions, such as crosslinking reactions, addition of initiator derived radicals to the double bonds, head-to-head coupling of carbon-centered radicals, and cross-termination between carbon-centered and thiol radicals.[30] Side reactions are favored by higher initiator concentrations,[30] as also shown for the here presented results. However, it should be emphasized that a low initiator amount is sufficient to promote an effective SH attachment. Therefore, for the next steps of the work, the amount of initiator was fixed to 1% (mol) relative to the amount of SH groups.

3.2. Covalent Conjugation of PGICL NPs with BSA

In a next step, BSA was chosen as model protein for the covalent conjugation of a protein on the surface of PGICL NPs via thiol-ene reaction. Therefore, BSA was modified through a reaction with the Traut’s reagent, in order to introduce free SH groups. This modification is necessary, as BSA has originally only one cysteine with a free thiol group per molecule.[31] After the modification, the final protein concentration was measured by the Pierce assay. Additionally, the number of thiol groups
was determined by the Ellman’s assay, which was an average of five thiol groups per BSA molecule.

As shown in Figure 3, flow cytometry and FCS measurements were performed in order to provide evidences of the covalent conjugation occurrence, by comparing the resistance of the conjugates to dissociation. Dissociation was induced by extensive centrifugation/washing cycles performed in order to remove unbound (or weakly adsorbed) BSA (items 2.4.2.2 and 2.4.2.3). In spite of its limitations for isolating proteins from nanoparticle-macromolecules complexes,[32,33] centrifugation is an uncomplicated separation method that has provided satisfactory results in many studies,[27,34] including to verify protein conjugation on nanoparticles.[10,35] For flow cytometry and FCS measurements, the conjugates were produced using the fluorescently labeled protein, BSA-FITC, which was also modified by Traut’s reagent before conjugation reactions.

Flow cytometry technique enables qualitative and semi-quantitative understanding of the nanoparticle bio-interface measurements.[36] In this study, this technique was used to give information about the percentage of particles that emitted fluorescent light after excitation by a 488 nm laser in each evaluated sample. Figure 3A shows FC results for uncoated NPs, non-covalent and covalent conjugates. The region evaluated (R1) has been adjusted to the upper right corner, which indicates

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**Figure 2.** A) Transmission electron microscopy (TEM) image of PGICL nanoparticles and information about particle size and zeta potential; B) Scheme representing the surface functionalization of PGICL nanoparticles with N-acetylcycteine (NAC) through thiol-ene reaction; C) Global double bond consumption and double bond consumption caused by SH group attachment. **Dp:** Particle diameter; **PDI:** polydispersity index; **ζ potential:** zeta potential.
fluorescent positive events (conjugates), while the large population in the lower left corner is based on non-fluorescent dust/debris (see Figure S1, Supporting Information). In addition, the median fluorescence intensity (MFI) of the fluorescent positive event was determined to compare the labeling efficiency of the different conjugates (see Figure S2, Supporting information). For non-covalent conjugates, around 26% of the nanoparticles emitted fluorescence (MFI = 253), while for covalent conjugates this value was around 36% (MFI = 278). Since the fluorescence of the particle is caused by the presence of BSA-FITC, this means that covalent conjugates remained with a higher amount of BSA-FITC on its surface after the centrifugation/washing cycles, in comparison to non-covalent conjugates. Comparable results were also reported for polystyrene particles, which were functionalized with fluorescently tagged albumin.\textsuperscript{[37]} A significantly higher amount of albumin could be immobilized on the nanoparticles’ surface via a covalent conjugation strategy in comparison to the physical adsorption.
Due to its very high sensitivity and selectivity, the fluorescence correlation spectroscopy is particularly suitable to study interactions between proteins and nanoparticles.\[38\] Here we used the technique to investigate the covalent and non-covalent attachment of BSA-FITC to the NPs. Typical FCS autocorrelation curves are shown in Figure 3B. The curve measured in a reference solution of BSA-FITC can be well represented by a single component fit, which yields a value of $3.3 \pm 0.3$ nm for the hydrodynamic radius of BSA, that is consistent with other studies.\[39\] The autocorrelation curve measured in solutions of the covalent conjugates is significantly shifted towards longer lag times confirming the presence of larger fluorescent species, that is, BSA-FITC nanoparticle conjugates with an average hydrodynamic radius of $60 \pm 6$ nm. This value is somewhat smaller than the one measured by DLS, probably as a consequence of the different kind of polydispersity averaging of the techniques.\[40\] Furthermore, the two component fit needed to represent this autocorrelation curve reveals that a small fraction ($\approx 10\%$) of nonattached, freely diffusing BSA-FITC is still remaining after the centrifugation/washing cycles. In the case of the non-covalent conjugates, the fraction of free BSA-FITC is almost 50\% indicating that these conjugates are less resistant to dissociation.

The data obtained by FCS are in agreement with the flow cytometry results, providing semi-quantitative evidences of the successful formation of covalent conjugates, which are more stable and resistant to dissociation.

DLS, $\xi$ potential and TEM imaging measurements were performed in order to characterize the conjugates regarding its size, stability, and morphology, respectively. The results are shown in Figure 4.

After covalent functionalization, the conjugates presented a very slight increase in the particle diameter, as expected, from $146 \pm 2$ nm (uncoated NPs—Figure 2A) to $154 \pm 3$ nm, probably caused by the formation of the protein layer around the NPs. $\xi$ potential values also suffered changes after NPs conjugation with BSA, slightly decreasing from $-54.1 \pm 0.3$ mV (uncoated NPs – Figure 2A) to $-62.0 \pm 0.8$ mV. This behavior was also observed by other authors\[41\] and it is attributed to the BSA charge contribution, since it has an overall negative charge at pH $> 5.5$.\[42\]

TEM images of uncoated NPs (images 1 and 3) and covalent conjugates (images 2 and 4), obtained by staining technique with uranyl acetate are presented. These images confirm the formation of a protein layer surrounding the nanoparticles. This layer contains proteins that even after purification remained tightly bonded to the surface of the NPs by covalent bonds or other strong interactions. Very similar behavior was found in previous reports.\[27,41\]

### 3.3. Cell Uptake

The uptake of uncoated NPs and BSA-NPs covalent conjugates into HeLa cells was analyzed by flow cytometry (Figure 5). NPs and BSA-NPs conjugates were applied to cell culture medium without additional proteins, supplemented with fetal bovine serum (FBS) or human serum. This way, it was possible to observe how conjugation with BSA affects NPs cell binding in different environments.

Flow cytometry analysis clearly indicates that, BSA conjugation reduced cell binding by HeLa cells and macrophages...
(see Figures S3 to S5, Supporting Information) under serum-free conditions. In literature serum albumin is referred to the protein class of dysopsonins,[43,44] meaning that adsorption of serum albumin to the nanoparticles’ surface can mask the cellular recognition by phagocytic cells. This process is known as “stealth effect”[2,45] and leads to a prolonged blood circulation time, enabling the nanoparticle to reach the desired target.[51,46]

Next to this, we observed that once NPs are introduced to cell culture medium with FBS or serum, cellular binding is even further reduced compared to serum-free conditions. This effect was observed for both non-functionalized and functionalized NPs. This means that next to BSA-surface functionalization, additionally serum proteins covering the NPs surface reduce cellular binding. This effect has also been observed for PEGylated nanoparticles, where it was shown that specific corona proteins (e.g., clusterin) hamper cellular recognition of nanoparticles.[2] Taking this together, it highlights that the covalent modification of the NPs surface with BSA is a feasible strategy to obtain stealth properties and hereby, increase the blood circulation time and improve the efficiency of treatments.

4. Conclusions

In the present work, PGICL nanoparticles were successfully conjugated with bovine serum albumin by thiol-ene reaction, producing BSA-nanoparticle conjugates, which are resistant to dissociation. Flow cytometry and FCS measurements revealed that the conjugates produced by BSA covalent attachment remained with a larger amount of proteins on its surface even after the centrifugation/washing cycles, indicating a higher stability in comparison to the conjugates produced by non-covalent method. The conjugates visualization was possible through TEM imaging, and a protein layer attached to the surface of the nanoparticles was clearly observed. Cell uptake assays indicated a reduced internalization of the nanoparticles by Hela cells and macrophages after conjugation with BSA. These results confirm that covalent BSA-nanoparticle conjugation is a feasible strategy to produce stable conjugates with stealth properties.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

The authors would like to thank Symrise (Brazil) for kindly supplying the monomer globalide, and Katrin Kirchhoff for the TEM images. The authors gratefully acknowledge CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) / Programa Doutorado CAPES-DAAD-CNPO / Processo nº 88887.1611406/2017-00, for the fellowship, and the financial support of German Science Foundation (SFB1066).

Conflict of Interest

The authors declare no conflict of interest.

Keywords

covalent conjugation, fluorescence correlation spectroscopy, nanoparticles, proteins, thiol-ene reaction

Received: April 26, 2019
Revised: August 16, 2019
Published online: September 6, 2019

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