Pharmaceutical nanotechnology

Folic acid-functionalized human serum albumin nanocapsules for targeted drug delivery to chronically activated macrophages

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ABSTRACT

Activated synovial macrophages play a key role in Rheumatoid Arthritis (RA). Recent studies have shown that folate receptor beta (FRβ) is specifically expressed by activated macrophages. Therefore a folate-based nanodevice would provide the possibility of delivering therapeutic agents to activated macrophages without affecting normal cells and tissues.

This study shows for the first time the sonochemical preparation of HSA nanocapsules avoiding toxic cross linking chemicals and emulsifiers used in other methods.

Production of HSA nanocapsules was optimized leading to a diameter of 443.5 ± 9.0 nm and a narrow size distribution indicated by a polydispersity index (PDI) of 0.066 ± 0.080. Nanocapsules were surface modified with folic acid (FA) and the FA content was determined to be 0.38 and 6.42 molecules FA per molecule HSA, depending on the surplus of FA employed. Dynamic light scattering was used to determine size, PDI and zetapotential of the produced nanocapsules before and after surface modification. FA distribution on the surface of HSA nanocapsules was localized three-dimensionally after fluorescence labeling using confocal laser scanning microscopy (CLSM). Furthermore, specific binding and internalization of HSA nanocapsules by FRβ-positive and FRβ-negative macrophages, obtained from human peripheral blood mononuclear cells, was demonstrated by flow cytometry. FRβ-expressing macrophages showed an increased binding for FA-modified capsules compared with those without FA.

1. Introduction

Rheumatoid Arthritis (RA) is a chronic, systemic autoimmune disorder that is characterized by inflammation of the joints. Chronically activated synovial macrophages play a key role in this inflammatory disease with numbers and level of macrophage activation correlating with the extent of joint inflammation and tissue degradation (Adamopoulos et al., 2006; Schett, 2008). Amongst others, some therapies are designed to eliminate the entire population of macrophages. However, this can cause severe side effects since macrophages have an essential function in fighting infectious diseases and promoting tissue repair. Consequently, specific elimination of the sub-population of chronic activated macrophages has been suggested as an alternative to the elimination of the entire macrophage population. Delivery of therapeutic agents selectively to pro-inflammatory cells would avoid toxicity and collateral damage to healthy cells. Recent studies have shown that the folate receptor beta (FRβ), which displays a high affinity for FA, is specifically expressed by activated macrophages (Puig-Kröger et al., 2009; Ross et al., 1999; Van Der Heijden et al., 2009). Therefore a folate-based nanodevice would provide the possibility of delivering therapeutic agents to activated macrophages without affecting normal cells and tissues.

Various types of folate conjugated drug delivery systems, such as liposomes (Gabizon et al., 1999), copolymer nanoparticles (Stella et al., 2000), baculovirus (Kim et al., 2007) and nanoparticles produced by HSA (Ulbrich et al., 2011) were previously described. Due to their uniquely diverse structures and functions, proteins are

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described to be highly interesting for the creation of new materials (Witus and Francis, 2011). HSA shows various valuable properties such as biodegradability, biocompatibility, effective drug loading capacity due to drug-binding properties of natural albumin and water solubility (Müller et al., 1996). HSA provides several functional groups on the surface which can be easily used for surface modification. Based on coupling to these functional groups, FA could be specifically attached onto the surface of the nanodevice rendering it recognizable by activated macrophages. However, many conventional techniques for the preparation of protein based nanocapsules involve cross-linking with toxic chemicals (Langer et al., 2003). Here we describe a strategy for the preparation of HSA nanocapsules which avoids toxic cross-linking chemicals such as glutaraldehyde commonly used in the desolvation.

Sonochemical methods have previously been described for the preparation of protein microspheres with an average diameter of 2.5 μm (Grinstaff and Suslick, 1991; Silva et al., 2011). However, it was reported that small nanoparticles were taken up more efficiently by the cells, when compared to such microparticles (Suri et al., 2007; Panyam and Labhasetwar, 2003). Likewise, in vivo experiments demonstrated that nanoparticles show a better performance since they diffused throughout the sub-mucosal layers while microparticles were predominantly localized in the epithelial lining (Desai et al., 1996). Consequently, in this study, stable FA-functionalized HSA nanocapsules in a size range of 500 nm were developed. The absence of crosslinking chemicals and emulsifiers in the production of these nanocapsules also provides an advantage from the economical point of view. Confocal laser scanning microscopy (CLSM) has the advantage over conventional light microscopy and scanning electron microscopy of gaining information about the three dimensional localization of fluorescence labeled compounds (Lamprecht et al., 2000). Thus, we used CLSM to monitor the reaction conditions and allow three-dimensional analysis of FA distribution on the surface of HSA capsules after fluorescence labeling of FA and HSA. Furthermore, specific internalization of FA modified nanocapsules was shown in primary human macrophages.

2. Materials and methods

2.1. Materials

Human serum albumin (HSA), folic acid (FA), N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDAC), N-hydroxysuccinimide (NHS), n-dodecane, dimethylformamide (DMF), fluorescein 5(6)-isothiocyanate (FITC) and fluorescent red mega 520 NHS ester (fluorescent red) were purchased from Sigma–Aldrich (Steinheim, Germany). All other reagents were used in analytical grade.

2.2. Methods

2.2.1. Fluorescent labeling

For CLSM studies FA and HSA were labeled with fluorescent dyes prior to capsule production. FA was labeled with fluorescent red. Therefore, 1 mg of FA was dissolved in 200 μL buffer (50 mM bicarbonate buffer, pH 9) and 150 μL of DMF were added. 1 mg of fluorescent red was dissolved in 50 μL DMF. 14 μL of fluorescence red stock solution (1 equiv.) were mixed with 70 μL of FA stock solution (1 equiv.). 100 μL of DMF were added and the mixture was shaken for 12 h at 25 °C. The mixture was purified by SEC using a HiTrap Desalting column (GE Healthcare Europe GmbH, Vienna, Austria) installed on an Äkta Purifier system (Amersham Pharmacia Biotech, Uppsala, Sweden), with 50 mM potassium phosphate and 100 mM NaCl (pH 7) as eluent, at the flow rate of 1 mL min^{-1} to

Table 1
Reaction conditions for the preparation of HSA nanocapsules by ultrasonication.

Sample	Treatment time [min]	Amplitude control [%]	Pulsation [%]
1	5	50	10
2	5	50	50
3	5	70	10
4	5	70	50
5	10	50	10
6	10	50	50
7	10	70	10
8	10	70	50
9	10	60	20

remove unlinked FA. Fractions which showed absorbance at 520 nm for fluorescent red and 368 nm for FA, respectively were collected.

For labeling with FITC, 4 mL of a HSA solution (10 mg mL^{-1} in bicarbonate buffer, pH 9.2) were mixed with 200 μL of FITC solution (5 mg mL^{-1} in DMSO). This mixture was shaken for 4 h at 25 °C under light protection. Thereafter the mixture was purified by SEC using a HiPrep 26/10 Desalting column (GE Healthcare Europe GmbH, Vienna, Austria) using the same settings like described above at a flow rate of 3 mL min^{-1} to remove excess FITC. Fractions showing absorbance at 280 and 485 nm were collected and reduced to a volume of 4 mL using a Vivaspin 30 kD centrifugal separator at 5000 rpm using Biofuge primo (Heraeus, Buckinghamshire, England).

2.2.2. Production of HSA nanocapsules

For nanocapsule production, a mixture of 50% FITC-labeled and 50% unlabeled HSA was used. 10 mL HSA-solution (2 mg mL^{-1} in 100 mM potassium phosphate buffer, pH 8) were combined with 6.6 mL of dodecane. This mixture was sonicated using a 13 mm disrupter horn connected to Branson Sonifier 250 on ice using the time, amplitude control and pulsation parameters as specified below (Table 1). Horn amplitudes are specified for certain amplitude control settings (21.0 μm for an amplitude control setting of 10%; 76.0 μm for 50%, 145.0 μm for 100%). A power of 70 W was required to maintain the amplitude for a given amplitude control setting of 50%. For phase separation the samples were stored 4 °C for 12 h. Thereafter, the organic phase was removed and the nanocapsules were washed three times with 100 mM sodium-phosphate buffer, pH 7.4 by centrifuging (5 min, 5000 rpm) and pouring away the supernatant. The solution was filtered through a 0.8 μm syringe filter to remove bigger capsules.

2.2.3. Surface modification

Coupling of FA on HSA nanocapsules: FA was coupled covalently on the surface of HSA nanocapsules in different concentrations. Stock solutions of FA, NHS and EDAC were prepared in 130 mM NaHCO$_3$ buffer (pH 7) as follows, FA: 5 mg mL^{-1}, NHS: 20 mg mL^{-1}, EDAC: 20 mg mL^{-1}. FA was mixed with 50 equiv. of EDAC and NHS and the mixture was activated for 20 min at 25 °C. Thereafter, the HSA nanocapsule solution was combined with different concentrations of activated FA (10 equiv., 50 equiv. to HSA concentration) and reacted for 12 h at room temperature. Subsequently, the capsules were separated from uncoupled FA by washing five times with ddH$_2$O using Vivaspin 30 kD at 5000 rpm.

2.2.4. Analysis

FA and HSA concentration: The concentration of FA in free conjugates was determined photometrically. A calibration curve of FA samples with concentrations from 1 to 500 μg mL^{-1} in 130 mM NaHCO$_3$ buffer (pH 7) was recorded at 368 nm. The HSA concentration was determined using Roti®-Nanoquant (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) which is based on the method of Bradford (Bradford, 1976). For the determination of FA and HSA

concentrations in capsules, samples were freeze dried and the dry powder was re-dissolved in 130 mM NaHCO$_3$ buffer (pH 7) to disintegrate capsules and give a solution of free HSA and HSA–FA molecules. Disintegration of capsules was followed by monitoring the number mean of particles using DLS. The FA concentration was determined by calculating the area of the absorbance peak at 368 nm and interpolation. For this purpose, a calibration curve was recorded with different concentrations of FA in the presence of HSA nanocapsules as background.

The interference of HSA and FA on the different analysis methods was tested. Mixtures of HSA and FA with known concentrations of both analytes were prepared and the concentrations were determined with the described methods. Measured and theoretical values were compared to confirm that FA and HSA have no influence on the analysis methods.

Confocal laser scanning microscopy: Surface modified HSA-capsules in the micron range were used as model to perform CLSM experiments with a Leica TCS SPE Confocal microscope (Leica Microsystems GmbH, Mannheim, Germany). Laser light wavelengths of 532 nm and 488 nm were used for the excitation of fluorescent red and FITC, respectively. The emitted light was detected in the range of 644–684 nm for fluorescent red and 500–556 nm for FITC. For every channel and every field of view, laser intensity, photomultiplier gain and offset were individually modified to optimize the signal/noise ratio. Confocal stacks were acquired with an ACS APO 63.0× 1.30 oil objective by applying a Z-step of 6.01 μm. Volume rendering and three dimensional models were created with the software Imaris7.0 (Bitplane, Zurich, Switzerland).

Dynamic light scattering (DLS): The mean size of HSA nanocapsules was determined by DLS using Zetasizer Nano ZS (Malvern Instruments GmbH, Herrenberg, Germany). Measurements were performed in triplicates at 25 °C with angle detection at 173°. Nanocapsules were diluted with 100 mM sodium-phosphate buffer, pH 7.4–0.1 mg mL^{-1}. Size distribution was characterized by the PDI. Zeta potential was determined based on a combination of laser Doppler velocimetry and phase analysis light scattering using Zetasizer Nano ZS.

2.2.5. Cell studies

Human peripheral blood mononuclear cells were isolated from buffy coats of healthy volunteers from the Instituto Português do Sangue (IPS, Porto, Portugal) by centrifugation over Lymphoprep (Axis-Shield) and monocytes were further purified by magnetic cell sorting using CD14 microbeads (Miltenyi Biotec). Monocyte-derived macrophages were generated in RPMI 1640 (supplemented with 10% FCS, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin) in the presence of 50 ng mL^{-1} M-CSF (Pepro Tech) for 7 days followed by an activation of 24 h with 20 ng mL^{-1} IL-4 (Pepro Tech).

The presence of surface FRβ in macrophages was evaluated by flow cytometry using a monoclonal FRβ antibody (Exbio, Prague, Czech Republic). Prior to antibody staining, Fc receptors were blocked with FcR Blocking Reagent (Miltenyi Biotec). An isotype-matched monoclonal antibody was used as negative control. Flow cytometry analysis was performed with a FACSCalibur flow cytometer (BD Biosciences) and data was analyzed using FlowJo software version 8.7 (Tree Star Inc.).

To obtain FRβ-positive and FRβ-negative macrophages, monocytes were incubated with 10 ng mL^{-1} GM-CSF (Pepro Tech) for 7 days. FRβ-positive and FRβ-negative macrophages were then separated using a FACSaria cell sorter (BD Biosciences).

Binding and internalization of HSA nanocapsules by macrophages was assessed by flow cytometry while all tests were performed in duplicates. Briefly, macrophages were incubated for 1.5 h with 0.1 mg mL^{-1} HSA nanocapsules in complete RPMI at 37 °C (binding and internalization allowed) or at 4 °C (binding only). After incubation cells were washed with PBS (pH 7.4) or with acidic NaCl 0.9% (pH 3). Data was acquired on a FACScalibur flow cytometer. The binding was determined as the geometrical mean fluorescence intensity of the HSA nanocapsules corrected for the background staining of cells not incubated with the nanocapsules. Mean fluorescence intensity equal or below 1.0 means no binding.

The viability of macrophages in the presence of the HSA nanocapsules was evaluated using the Resazurin assay. This method is based on the ability of living cells to convert the dye Resazurin into a fluorescent product (Resorufin). M-CSF-differentiated macrophages were incubated with different concentrations of HSA-nanocapsules for 24 h at 37 °C, followed by incubation with Resazurin for 4 h. Fluorescence was then read at 560/590 nm in a plate-reading fluorometer (SpectraMax Gemini XS, Molecular Devices).

3. Results and discussion

3.1. Preparation of HSA nanocapsules

A sonochemical method for the production of FA-functionalized HSA nanocapsules was developed. The strategy presented here avoided the use of toxic chemicals for cross-linking and aimed at a narrow size distribution to allow delivery of therapeutic agents to activated macrophages without affecting normal cells and tissues.

A commonly used method for the preparation of albumin nanoparticles is the desolvation technique (Langer et al., 2003). Using this method, nanoparticles in a size range between 150 and 280 nm can be prepared. A disadvantage of this method is the use of toxic glutaraldehyde as crosslinker to stabilize the prepared nanoparticles. The use of toxic cross-linkers for the preparation of protein aqueous microspheres can be avoided by using sonochemical strategies. However, previously capsules in a size range of 2.5 μm were achieved (Grinstaff and Suslick, 1992), which is far too big in order to allow for specific cell internalization. Particles in this size range are recognized by macrophages as foreign particles and are consequently taken up through phagocytosis (Ahsan et al., 2002). This would not only lead to elimination of the particles before showing a therapeutic effect, but would also affect healthy macrophages. In non-phagocytic cells nanoparticles with a size of 100 nm showed a relatively higher intracellular uptake compared to microparticles (Suri et al., 2007; Panyam and Labhasetwar, 2003). Therefore, a specific non-phagocytic uptake due to recognition of folic acid by the FRβ can only be achieved with nanoparticles. Size is also an important factor for in vivo experiments since it was shown that 100 nm particles diffused throughout the sub-mucosal layers while microparticles were predominantly localized in the epithelial lining (Desai et al., 1996).

In this study, optimization of energetic and time parameters in ultrasound-based formation of nanocapsules lead to an average diameter of below 500 nm and a narrow size distribution. Nine combinations of amplitude, pulsation and total treatment time were tested while the pH value was fixed to pH 8, which was previously demonstrated to be the optimum for protein nanoparticle production (Sebak et al., 2010).

DLS measurements of HSA-capsules after several washing steps showed a broad size distribution indicated by a PDI value around 0.3 in all samples. Data analysis proved that two populations of particles with diameters around 300 nm and around 900 nm, respectively, were obtained. Consequently, a filtration step was incorporated to eliminate the larger diameter particle population. Testing filter pore sizes of 1.0 μm and 0.8 μm, with the smaller pore size particles larger than 800 nm were successfully eliminated.
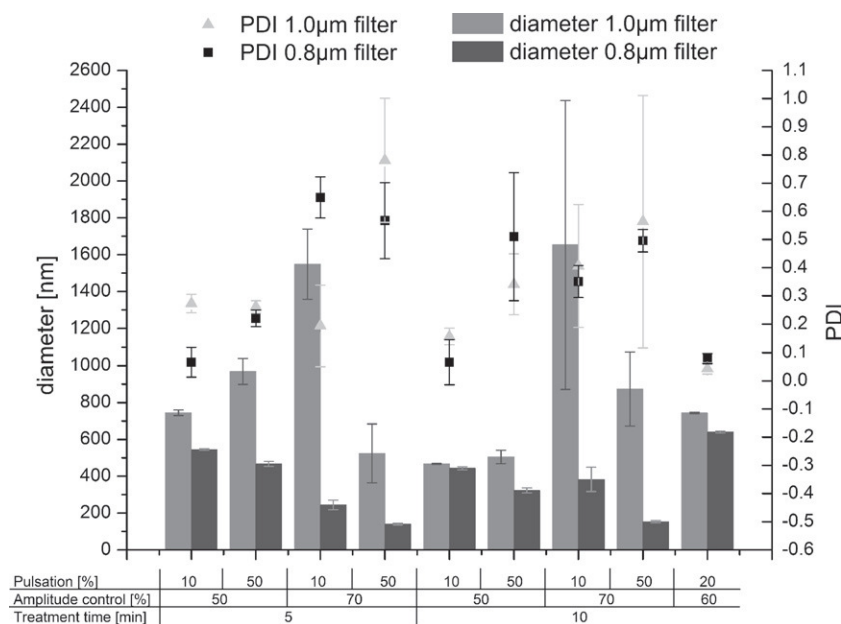
Fig. 1. Parameter (pulsation, amplitude control, treatment time) optimization of ultrasonic production of HSA nanocapsules towards small diameters and PDI (polydispersity index). Diameter and PDI were measured after filtration (pore size 1.0 and 0.8 μm).

Moreover, a narrow size distribution with minimum PDI values of around 0.07 was obtained.

Thus, in all subsequent experiments an amplitude control of 50%, pulsation 10% and 10 min treatment time for sonication were used. These optimized energetic and time parameters gave small nanocapsules with a diameter of 443.5 ± 9.0 nm and a narrow size distribution indicated by a PDI of 0.066 ± 0.080 (Fig. 1).

3.2. Surface modification

For recognition by activated macrophages, FA was attached to the surface of the nanocapsules via the carboxylic end of the molecule, since the pteridine residue is responsible for FRβ recognition and therefore cannot be modified. The water soluble, heterobifunctional cross-linker EDAC was used to activate FA-carboxylic groups which further react with primary amines located on the capsule surface.

Preliminary results indicated that disintegration of nanocapsules was necessary prior to spectroscopic quantification of FA at 368 nm and HSA quantification based on a modified Bradford assay. To exclude any interference of HSA and FA absorption, calibration curves for FA quantification at 368 nm were recorded in the presence of disintegrated HSA-nanocapsules as background. Disintegration of nanocapsules was successfully demonstrated by using DLS analysis showing a lower number mean of 73.39 ± 30.98 nm and a PDI of 0.475 ± 0.186.

Capsules with two different concentrations of FA were produced to compare the cell internalization ability as a function of FA amount linked on the surface. The FA content on capsules was determined to be 0.38 and 6.42 molecules FA per molecule HSA respectively (Table 2). A maximum coupling yield of around 12.8%

was achieved when a 50 fold molar excess of FA was used for the coupling experiment.

3.2.1. Zetapotential, size, PDI

Size and PDI measurements showed that filtration is the essential step to obtain nanocapsules with a narrow size distribution. After filtration only nanocapsules with a diameter of around 500 nm and a PDI of 0.2 (Fig. 2) were found. An increase in polydispersity could be observed with higher amounts of FA linked on the capsule surface. This effect can be correlated with results from CLSM analysis where some inhomogeneity regarding capsule modification was seen. The zetapotential of the nanocapsules was around −20 mV and only a slight deviation could be observed after FA modification. With CLSM analysis it was verified that no aggregate formation occurred, even though it has been previously described that a zetapotential above (±)30 mV was the balance point between stable and unstable suspensions (Mohanraj and Chen, 2006).
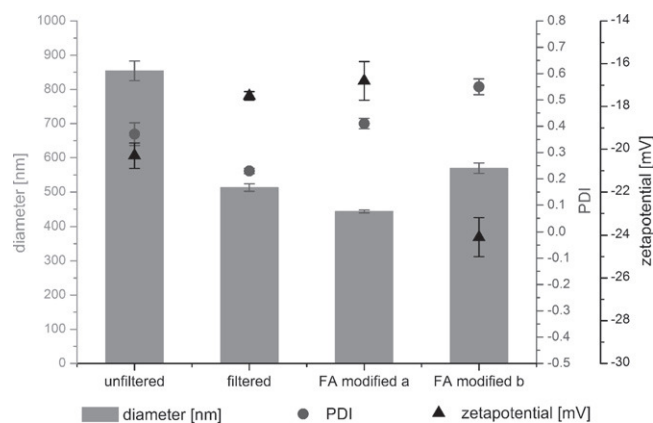
Table 2
Concentration of FA on nanocapsules calculated as molar ratio of FA molecules per HSA molecules used for the coupling reaction vs. FA concentration determined after coupling reaction.

Amount FA per HSA [mol mol^{-1}]	FA linked on HSA [mol mol^{-1}]
10	0.38 ± 0.02
50	6.42 ± 0.18

Fig. 2. Diameter [nm], PDI and zetapotential [mV] of HSA nanocapsules before and after filtration (pore size 0.8 μm) and after surface modification with FA in different molar excess (a) 0.38 mol FA per mol HSA and (b) 6.42 mol FA per mol HSA, both filtered.
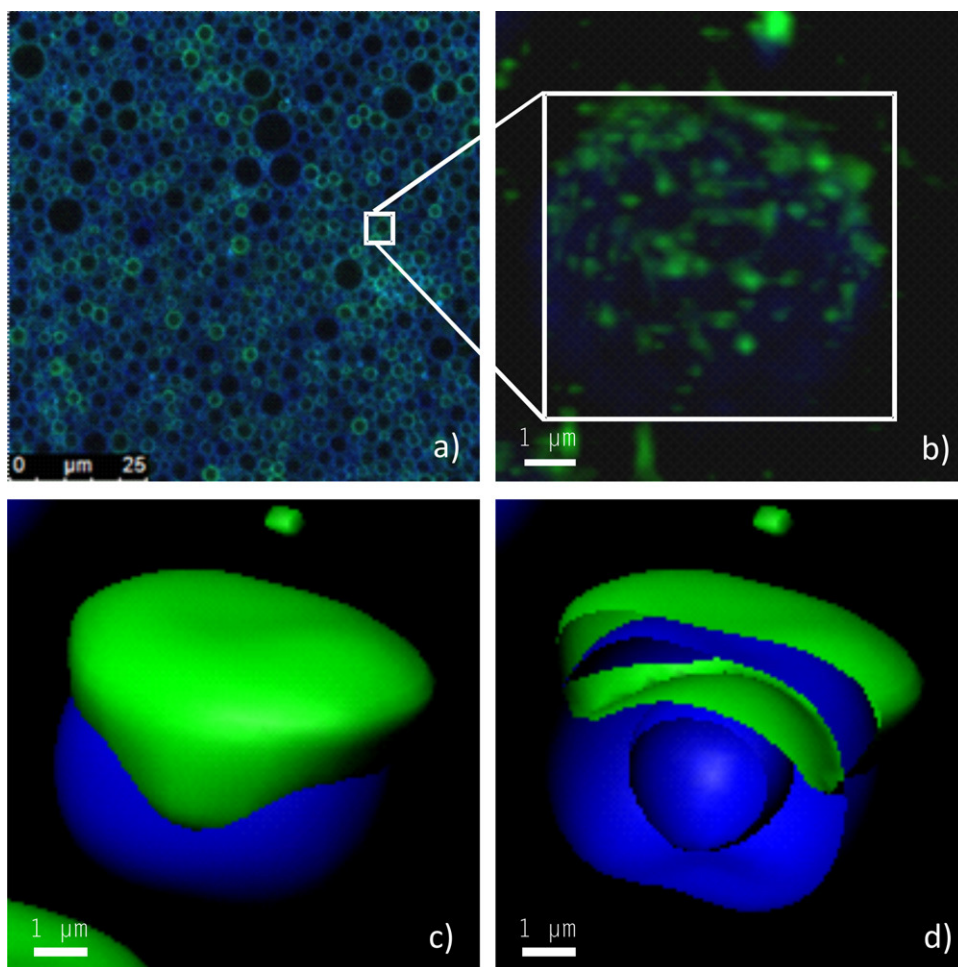
Fig. 3. CLSM image (a) and 3D reconstruction (b–d) of FITC-labeled HSA nanocapsules carrying fluorescent red labeled FA on the capsule surface. Blue: HSA, green: FA. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)
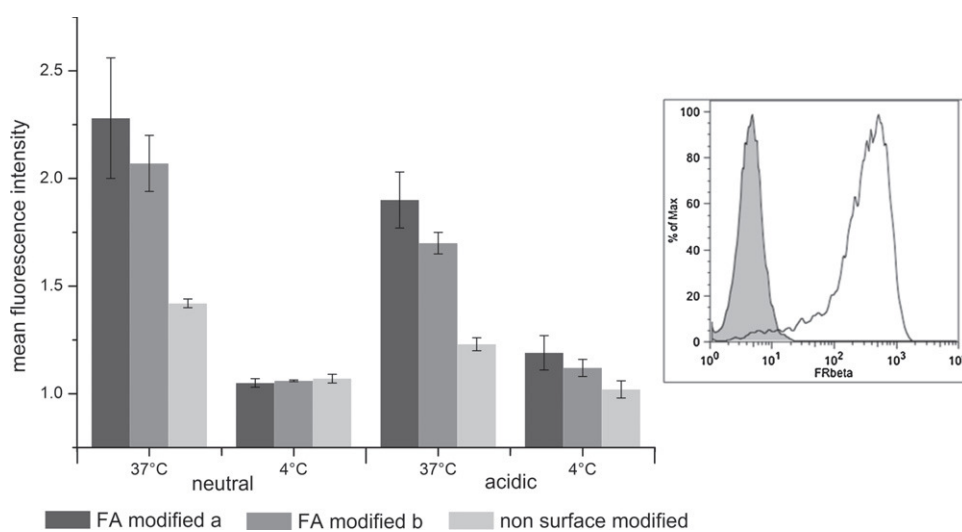

Fig. 4. (Left) Binding/internalization of FITC-conjugated HSA nanocapsules FA (a) 0.38 FA per HSA [mol mol^{-1}], FA (b) 6.42 FA per HSA [mol mol^{-1}] by macrophages after 1.5 h incubation at 37 °C or 4 °C followed by neutral or acidic washing. The binding was determined by flow cytometry as the geometrical mean fluorescence intensity of the HSA nanocapsules. Mean fluorescence intensity equal or below 1.0 means no binding. (Right) FRβ expression (flow cytometry analysis) in human activated macrophages. Macrophages were obtained by incubation with 50 ng mL^{-1} M-CSF for 7 days followed by 1 day of activation with 20 ng mL^{-1} IL-4. Solid black line represents FRβ expression and filled histogram represents the isotype control.

3.2.2. CLSM characterization

Amongst other methods, CLSM was used for the characterization of surface modified HSA capsules. This method is a powerful, nondestructive visualization technique for the characterization and visualization of structures on the surface and inside of particles (Lamprecht et al., 2000). Therefore, HSA capsules in micron range were prepared as a model for nanocapsules. HSA and FA were labeled prior to capsule production with the fluorescent dyes FITC and fluorescent mega red 520 NHS-ester, respectively.

No aggregate formation occurred between capsules, single capsules in micron range were clearly visible (Fig. 3). Using this method we observed that within the same batch some single capsules were completely modified with FA while others did not show any FA derived fluorescence signal (Fig. 3). Albert et al. (1952) had already described in 1952 that pteridines have the ability to form strong intermolecular hydrogen bonds through amino- and hydroxyl substituents and the heterocyclenitrogens. Due to the fact that FA contains the structural element of pteridine this phenomenon could be explained by the strong intermolecular attraction of FA molecules.

The three dimensional reconstruction of a single capsule showed a shell of HSA that is surrounding a cavity into which drugs could be loaded. FA is clearly located on the surface of the HSA capsule. In the reconstruction image FA is not completely surrounding the capsule. This is most likely an artifact due to signal loss of fluorescent red by high laser energy input during the scanning process ("photo bleaching" effect).

3.3. Cell studies

Previous studies have shown that macrophage activation plays a significant role in the pathogenesis of RA (Schett, 2008; Xia et al., 2009). The expression of FRβ by activated macrophages in the inflamed sites of RA (Turk et al., 2002; Van Der Heijden et al., 2009; Nakashima-Matsushita et al., 1999), allows the use of this receptor as a target for the specific delivery of therapeutic agents. In the present study, we evaluated the binding and internalization of FA–HSA nanocapsules (a potential folate-conjugated drug delivery system) by human FRβ-expressing macrophages using flow cytometry. FRβ-expressing macrophages (Fig. 4) showed an increased binding for capsules with FA when compared with those without FA (Fig. 4). Moreover, the observed binding recedes when incubation occurs at 4 °C when the internalization is not allowed (Fig. 4), suggesting that capsules are being internalized by macrophages. This conclusion is supported by the results observed after the acidic vs. neutral wash of macrophages incubated with capsules. Since the acidic wash eliminates any surface-bound capsules while the neutral wash only washes away unbound capsules, and no difference in the mean fluorescence intensity was observed between the two conditions (Fig. 4), we conclude that the observed fluorescence is due to internalized capsules. No significant difference was observed between nanocapsules with 0.38 and 6.42 molecules FA per molecule HSA, when nanocapsules with different amounts of FA linked on the capsule surface were compared. This suggests that a maximum of 0.38 molecules FA per HSA molecule is sufficient for efficient FA–HSA targeting of macrophages.

The specificity of FA–HSA nanocapsules for FRβ-expressing macrophages was further evaluated by incubating FRβ-positive and FRβ-negative macrophages with FA–HSA nanocapsules containing 0.38 molecules FA per molecule HSA. The internalization of the FA-nanocapsules was higher in FRβ-positive macrophages (mean fluorescence intensity of 2.58 ± 0.68) than in macrophages not expressing FRβ (mean fluorescence intensity of 1.67 ± 0.15), indicating that the binding/internalization of capsules is mediated through folate receptors. It has been reported that the reduced folate carrier, another protein used for folate transport in cells,
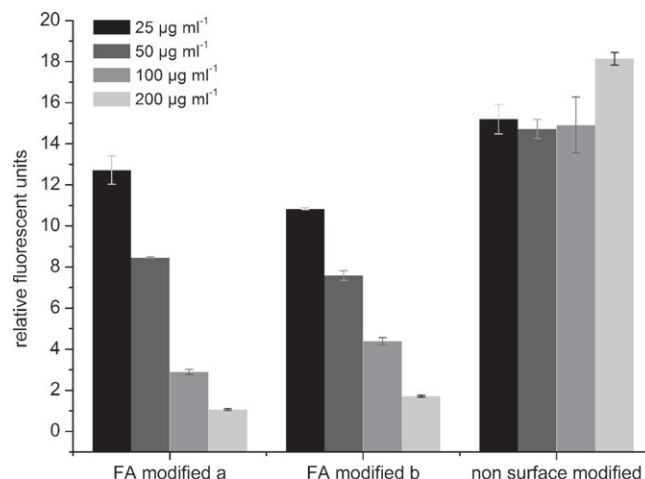

Fig. 5. Viability of macrophages in the presence of different concentrations of HSA nanocapsules after incubation time of 24 h. Viability was analyzed using the resazurin assay and it is expressed as Relative Fluorescent Units.

does not transport folate conjugates (Paulos et al., 2004) such as the capsules developed in this study, therefore the internalization of FA–HSA nanocapsules must be mediated through FRβ.

The viability of macrophages in the presence of nanocapsules was also evaluated. The incubation of macrophages with FA-modified HSA nanocapsules reduced viability in a concentration-dependent manner (Fig. 5). No toxicity was observed for HSA capsules (Fig. 5), as these are not internalized by macrophages (Fig. 4).

4. Conclusion

This work demonstrates the production of stable, uniform (size) HSA nanocapsules avoiding toxic crosslinking agents and emulsifiers. HSA nanocapsules in a size range of 440 nm were surface modified using different amounts of FA to allow for specific cell internalization by FRβ positive macrophages. Size, PDI and zetapotential were determined before and after surface modification. Using CLSM analysis it was found that FA modification is not uniform due to strong intermolecular attraction of FA molecules. Furthermore specific binding and internalization of FA modified HSA-nanocapsules by FRβ-positive macrophages was achieved in comparison with non-modified nanocapsules. In addition, the internalization of the FA-modified nanocapsules was three-fold higher in FRβ-positive macrophages than in macrophages not expressing FRβ, showing that the binding/internalization of capsules is being mediated through folate receptors. Further studies should include an internalization evaluation using other cell types to confirm specificity, as well as surface modification with other target molecules to enhance specificity of macrophage internalization. The folate based nanodevice produced during this study could be used to deliver therapeutic agents to activated macrophages without affecting normal cells and tissues. Consequently, future investigation should focus on loading the nanodevices with different drugs suitable for the treatment of RA.

References

Adamopoulos, I.E., Sabokbar, A., Wordsworth, B.P., Carr, A., Ferguson, D.J., Athanasou, N.A., 2006. Synovial fluid macrophages are capable of osteoclast formation and resorption. J. Pathol. 208, 35–43.

Ahsan, F., Rivas, I.P., Khan, M.A., Torres Suarez, A.I., 2002. Targeting to macrophages: role of physicochemical properties of particulate carriers – liposomes and microspheres – on the phagocytosis by macrophages. J. Control. Release 79, 29–40.

Albert, A., Brown, D.J., Cheeseman, G., 1952. 812. Pteridine studies. Part III: the solubility and the stability to hydrolysis of pteridines. J. Chem. Soc., 4219–4232.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Desai, M.P., Labhasetwar, V., Amidon, G.L., Levy, R.J., 1996. Gastrointestinal uptake of biodegradable microparticles: effect of particle size. Pharm. Res. 13, 1838–1845.

Gabizon, A., Horowitz, A.T., Goren, D., Tzemach, D., Mandelbaum-Shavit, F., Qazen, M.M., Zalipsky, S., 1999. Targeting folate receptor with folate linked to extremities of poly(ethylene glycol)-grafted liposomes: in vitro studies. Bioconjug. Chem. 10, 289–298.

Grinstaff, M.W., Suslick, K.S., 1991. Air-filled proteinaceous microbubbles: synthesis of an echo-contrast agent. PNAS 88, 7708–7710.

Grinstaff, M.W., Suslick, K.S., 1992. Proteinaceous microspheres. Am. Chem. Soc., 218–226.

Kim, Y.K., Choi, J.Y., Yoo, M.K., Jiang, H.L., Arote, R., Je, Y.H., Cho, M.H., Cho, C.S., 2007. Receptor-mediated gene delivery by folate-PEG-baculovirus in vitro. J. Biotechnol. 131, 353–361.

Lamprecht, A., Schäfer, U., Lehr, C.M., 2000. Structural analysis of microparticles by confocal laser scanning microscopy. AAPS PharmSci 1, 10–19.

Langer, K., Balthasar, S., Vogel, V., Dinauer, N., von Briesen, H., Schubert, D., 2003. Optimization of the preparation process for human serum albumin (HSA) nanoparticles. Int. J. Pharm. 257, 169–180.

Mohanraj, V.J., Chen, Y., 2006. Nanoparticles – a review. Trop. J. Pharm. Res. 5, 561–573.

Müller, B.G., Leuenberger, H., Kissel, T., 1996. Albumin nanospheres as carriers for passive drug targeting: an optimized manufacturing technique. Pharm. Res. 13, 32–37.

Nakashima-Matsushita, N., Homma, T., Yu, S., Matsuda, T., Sunahara, N., Nakamura, T., Tsukano, M., Ratnam, M., Matsuyama, T., 1999. Selective expression of folate receptor β and its possible role in methotrexate transport in synovial macrophages from patients with rheumatoid arthritis. Arthritis Rheum. 42, 1609–1616.

Panyam, J., Labhasetwar, V., 2003. Biodegradable nanoparticles for drug and gene delivery to cells and tissue. Adv. Drug Deliv. Rev. 55, 329–347.

Paulos, C.M., Turk, M.J., Breur, G.J., Low, P.S., 2004. Folate receptor-mediated targeting of therapeutic and imaging agents to activated macrophages in rheumatoid arthritis. Adv. Drug Deliv. Rev. 56, 1205–1217.

Puig-Kröger, A., Sierra-Filardi, E., Dominguez-Soto, A., Samaniego, R., Corcuera, M.T., Gomez-Aguado, F., Ratnam, M., Sanchez-Mateos, P., Corbi, A.L., 2009. Folate receptor β is expressed by tumor-associated macrophages and constitutes a marker for M2 anti-inflammatory/regulatory macrophages. Cancer Res. 69, 9395–9403.

Ross, J.F., Wang, H., Behm, F.G., Mathew, P., Wu, M., Booth, R., Ratnam, M., 1999. Folate receptor type β is a neutrophilic lineage marker and is differentially expressed in myeloid leukemia. Cancer 85, 348–357.

Schett, G., 2008. Review: immune cells and mediators of inflammatory arthritis. Autoimmunity 41, 224–229.

Sebak, S., Mirzaei, M., Malhotra, M., Kulamarva, A., Prakash, S., 2010. Human serum albumin nanoparticles as an efficient noscapine drug delivery system for potential use in breast cancer: preparation and in vitro analysis. Int. J. Nanomed. 5, 525–532.

Silva, R., Ferreira, H., Cavaco-Paulo, A., 2011. Sonoproduction of liposomes and protein particles as templates for delivery purposes. Biomacromolecules 12, 3353–3368.

Stella, B., Arpicco, S., Peracchia, M.T., Desmaele, D., Hoebeke, J., Renoir, M., D'Angelo, J., Cattel, L., Couvreur, P., 2000. Design of folic acid-conjugated nanoparticles for drug targeting. J. Pharm. Sci. 89, 1452–1464.

Suri, S., Fenniri, H., Singh, B., 2007. Nanotechnology-based drug delivery systems. J. Occup. Med. Toxicol. 2, 16.

Turk, M.J., Breur, G.J., Widmer, W.R., Paulos, C.M., Xu, L.C., Grote, L.A., Low, P.S., 2002. Folate-targeted imaging of activated macrophages in rats with adjuvant-induced arthritis. Arthritis Rheum. 46, 1947–1955.

Ulbrich, K., Michaelis, M., Rothweiler, F., Knobloch, T., Sithisarn, P., Cinatl, J., Kreuter Jr., 2011. Interaction of folate-conjugated human serum albumin (HSA) nanoparticles with tumour cells. Int. J. Pharm. 406, 128–134.

Van Der Heijden, J.W., Oerlemans, R., Dijkmans, B.A.C., Qi, H., Laken, C.J.V., Lems, W.F., Jackman, A.L., Kraan, M.C., Tak, P.P., Ratnam, M., Jansen, G., 2009. Folate receptor β as a potential delivery route for novel folate antagonists to macrophages in the synovial tissue of rheumatoid arthritis patients. Arthritis Rheum. 60, 12–21.

Witus, L.S., Francis, M.B., 2011. Using synthetically modified proteins to make new materials. Acc. Chem. Res. 44, 774–783.

Xia, W., Hilgenbrink, A.R., Matteson, E.L., Lockwood, M.B., Cheng, J.X., Low, P.S., 2009. A functional folate receptor is induced during macrophage activation and can be used to target drugs to activated macrophages. Blood 113, 438–446.