Doubly derivatized poly(lactide)–albumin nanoparticles as blood vessel-targeted transport device for magnetic resonance imaging (MRI)

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Abstract Molecular imaging using magnetic resonance imaging (MRI) is expected to play a crucial future role in oncological diagnosis and in monitoring of therapeutic progress. Targeted nanoparticle contrast media (CM) with high relaxivities are required in order to obtain adequate signal-to-noise ratios as well as visualization of a desired pathologic area of the human body. The aims of this study were to synthesize and define certain physicochemical and enhancement properties of new doubly derivatized polylactic acid–bovine serum albumin (PLA-BSA) nanoparticles (NPs) modified by the covalent coupling of glutaraldehyde as a crosslinking agent. An additional functionalization with endothelial cells (ECs) targeting groups (tomato lectins; LEA) and signal-emitting moieties (DTPA-Gd) enables its use as a macromolecular, biodegradable contrast agent for MRI. The NPs were characterized by different spectroscopies, size exclusion chromatography, and scanning and transmission electron microscopy. In a human vein model, the dynamics of the nanoparticle interactions with the vein wall were examined in MRI, with correlative imaging in electron microscopy. In vitro studies were conducted to show endothelial binding and persistent enhancement at the apical EC surface. NPs with a
diameter between 55 and 75 nm, able to carry simultaneous signal emitting, and targeting motifs on a single construct were successfully prepared. A high Gd payload and endothelial binding to blood vessel walls were observed. The binding affinity and specificity of LEA was preserved, and a strong enhancement at the endothelium was achieved. The stabilized core–shell structure of PLA-NP might allow for further encapsulation of lipophilic drugs or for attachment of other targeting molecules, such as antibodies.

**Keywords** Poly(lactide)-albumin · Tomato lectin · Nanoparticles · Endothelial cells · Targeting · MRI

**Introduction**

Small molecular weight gadolinium-based (Gd\(^{3+}\)) complexes (SMC) are the most applied contrast agents for magnetic resonance imaging (MRI) (Zhou and Lu 2013). After intravenous administration, SMC are distributed throughout the intravascular and interstitial space and are excreted rapidly via renal filtration (Runge 2000; Zhou and Lu 2013). They are well suited for delineating abnormal transendothelial permeability in the normally impenetrable blood–brain barrier. However, their normal unrestricted diffusion from the blood into the extracellular fluid (ECF), excluding the central nervous system, makes it difficult to detect abnormal vessel permeabilities associated with different pathologic states (Zhou and Lu 2013). The normally rapid transvascular distribution also makes SMC poorly suited for quantitative assessments of blood volume, as would be necessary for tumor characterization or for morphologic stress testing of the myocardium (Lohrke et al. 2016). Another limitation of SMC is the fact that they retain no tissue or organ specificity (Runge 2000). Specific targeting of tissues and organs would provide a strong and prolonged vascular enhancement from only the desired region of interest.

Nanoparticles (NPs) offer a wide variety of properties to overcome the limitations of SMC. Their specific pharmacokinetic, biodistribution, and target site accumulation have been attracting considerable interest, from drug and gene delivery to imaging, sensing of specific biomolecules or biostructures, and diagnostics. Certain features of NPs, such as multi-functionality, multivalence, and the ability to carry large payloads, have made them the subject of intense research. It has been demonstrated that NPs can be targeted effectively within living organisms; can emit signals for use in nuclear medical, radiological, and optical imaging systems; and can be used as drug carriers that release drugs by diffusive mechanisms (Debbage et al. 2001; Huang et al. 2010; Keshhtkar et al. 2018; Kessinger et al. 2011; Liu et al. 2017; Lohrke et al. 2016; Moradi Khaniabadi et al. 2017; Payne et al. 2017; Quan et al. 2011; Xie et al. 2009, 2010a, b). These proof-of-principle studies require more development that could combine these functional properties within single nanoparticles, and this task is now one of the highest priorities in nanomedicine.

The development of NPs-based contrast agents for MRI that are capable of prolonged dwell times in the human organism and provide long acquisition times, as well as specific crossing of intact tissue barriers (tissue and organ targeting), is greatly needed. Moreover, NPs made of biopolymers or proteins possess the most suitable characteristics for encapsulation of many drugs and diagnostic (imaging) agents, such as biodegradability and high loading capacity.

The formation of new blood vessels during the development and progression of cancer or atherosclerosis involves the sprouting, migration, and proliferation of endothelial cells (ECs). Visualization of ECs is crucial, and it will allow a detailed insight into pathophysiological processes as ECs underline every single blood vessel in the living organism (Debbage et al. 2001). MRI of the vascular network using ECs-targeted NPs may improve early diagnosis and therapy (Debbage et al. 2000; Moradi Khaniabadi et al. 2017).

The aims of this study were to synthesize and define certain physicochemical and MRI enhancement properties of new doubly derivatized polylactic acid–bovine serum albumin (PLA-BSA) nanoparticles (NPs) modified by covalent coupling of glutaraldehyde as a crosslinking agent. An additional aim was to functionalize the (PLA-BSA) NPs with both ECs targeting groups (tomato lectins; *Lycopersicon esculentum agglutinin*, LEA) and signal-emitting moieties (diethylenetriaminepentaacetic acid–gadolinium, DTPA-Gd) to enable their use as an EC-specific, macromolecular contrast agent for MRI.

*In vitro* MRI studies were conducted to show whether Gd-LEA-BSA-Gd-PLA causes endothelial binding to blood vessel walls with a strong, persistent enhancement predominantly at the apical EC surface.
Materials and methods

Materials

LEA was isolated and purified as described previously (Paschkunova-Martic et al. 2005). Poly (D,L-lactide; molecular weight 75,000–120,000), BSA (fatty acid free, essentially globulin free, lyophilized powder ≥98%), nitriloacetic acid trisodium salt (Na_3C_6H_6NO_6), CH_2Cl_2, and glutaraldehyde (50 % w/v) were purchased from Sigma-Aldrich, Austria. Gadolinium-DTPA (“Magnevist”) was bought from Schering (Berlin, Germany). Gd_2O_3 (purity >99 %) was provided by Dr. Peter Unfried, Institute of Inorganic Chemistry at the University of Vienna.

Methods

Preparation of Gd-LEA-BSA-Gd-PLA nanoparticles

Synthesis of PLA NPs with surface-attached BSA and BSA-Gd

An adapted emulsification method reported by Montisci et al. was applied to produce polylactide nanoparticles with bovine serum albumin as a hydrophilic surfactant (Montisci et al. 2001; Paschkunova-Martic et al. 2005). Two different preparations were carried out in parallel. Thus, 260.0 mg of PLA was dissolved in 5 ml of CH_2Cl_2 and emulsified in (a) 80 ml of an aqueous solution of BSA (20% w/v) and (b) 80 ml of BSA-Gd in water (2% w/v) (preparation of the BSA-Gd conjugate was reported previously using Heidolph DIAX 900 at 24,000 rpm, 3 min) (Paschkunova-Martic et al. 2005). The emulsions were stirred overnight at room temperature. Nanoparticles were collected by centrifugation (3300 × g, 15 min) and washed six times with water (Rotislov HPLC grade, ROTH). The suspensions were stored at 4°C. Plain BSA-PLA nanoparticles as a control were synthesized in the same manner without labeling with LEA or Gd.

Coupling of LEA

As next, LEA was covalently linked to BSA or BSA-Gd, anchored to the surface of the nanoparticles using glutaraldehyde as reported previously (Montisci et al. 2001; Paschkunova-Martic et al. 2005). In this study, we focused on the BSA-Gd-PLA NPs, which provide a first Gd layer of the nanoparticulate construct. The NPs were first activated and then incubated with the LEA: 50 mg BSA-Gd-PLA nanoparticles were washed twice with phosphate-buffered saline (PBS 10 mmol, pH 7.4, by centrifugation, 3300 × g, 10 min). The pellet was resuspended by vortexing in 1 ml of PBS and then glutaraldehyde (1 ml, 25 %) was added. The mixture was shaken gently for 6 h to activate the amino groups. The suspension was centrifuged to remove unreacted glutaraldehyde and was washed three times in phosphate-buffered saline (PBS 10 mmol, pH 7.4) to remove any traces of glutaraldehyde that might crosslink the lectin molecules. Subsequently, 900 μl of PBS containing 250 μg of lectins was added and the linkage was achieved by incubation overnight at room temperature. The LEA-BSA-Gd-PLA conjugates were centrifuged to remove free lectins and finally resuspended in 1 ml PBS.

Conjugation with diethylenetriamine pentaacetic acid bisanhydride (DTPABA)

A 220-fold excess of DTPABA (3.5 mg, 0.001 mmol) was suspended in 0.028-ml DMSO and added step-wise to the LEA-BSA-Gd-PLA solution. After each portion, the pH was adjusted to 8.5 with 3 M NaOH. The reaction mixture was then incubated 1 h at RT and vortexed every 10 min.

Complexation with gadolinium (Gd^{3+})

The above-prepared sample was centrifuged and the supernatant was purified on a fast protein liquid chromatography (FPLC) system to isolate the unbound DTPABA and other low molecular weight substances. FPLC purification was carried out as described by us earlier (Paschkunova-Martic et al. 2005; Stollenwerk et al. 2010). The protein fraction, as well as the pellet, which was resuspended in citrate buffer (100 mmol, pH 6.5) was further complexated with Gd(NTA)_2 (0.060 ml, 116.0 mmol) was added and stirred for 24 h at 4 °C. Subsequently, the solutions were lyophilized overnight and then dissolved in 0.5 ml of distilled water. The samples were purified and desalted using the FPLC system to separate unbound Na_3Gd(NTA)_2, buffer salts, and other low molecular weight compounds. Subsequently, the isolated protein fractions were again lyophilized.

Core–shell structured nanoparticles with hydrophobic PLA chains in-oriented and two layers of each BSA-Gd and LEA-Gd conjugates comprising the shell were found in the supernatant, as well as in the pellet (Figs. 1 and 2).
Physico-chemical characterization of the Gd-LEA-BSA-Gd-PLA nanoparticles. PLA hydrophobic chains orientate to NP core. Hydrophilic BSA-Gd layer absorbed onto NPs, being covalently stabilized and linked via glutaraldehyde to LEA.

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The final Gd-LEA-BSA-Gd-PLA NPs were analyzed after purification by FPLC to check Gd payload, albumin, and lectin content. The methods used for these determinations were AAS (atom absorption spectrometry) to estimate metal concentration and different protein assays as described previously (Paschkunova-Martic et al. 2005; Stollenwerk et al. 2010). For protein (BSA) or glycoprotein (LEA) determination, either the classic Bradford method or the Amidoblack method was applied, respectively. For each synthetic run, the BSA or LEA content of nanoparticles was quantified by photometrical determination. Briefly, to determine LEA concentration, the Amidoblack method for the determination of glycoproteins was used. The samples were incubated with a dye solution and washed several times, and then, a photometrical determination was carried out at \( \lambda = 625 \) nm. The calibration curve was created using BSA solutions of different concentrations. The classic Bradford assay was applied to determine BSA content in the nanoparticles. This method is based on the idea that the absorption of an acidic solution of Coomassie Brilliant Blue G250 (CBB-G250) can be shifted from 465 to 595 nm via protein binding. The absorption of the BSA-CBB from the nanoparticles was measured at 595 nm, and the concentration of the samples was determined using a calibration curve.

Gadolinium quantification was carried out using a Z 5100 Zeeman atomic absorption spectrometer (Perkin-Elmer) equipped with a furnace atomiser (Perkin-Elmer HGA60), a Gd hollow-cathode lamp (Perkin-Elmer), and an automatic sampler (Perkin-Elmer AS-60).

**ATR-FTIR** (attenuated total reflectance–Fourier transform infrared spectroscopy) spectra of the NPs’ components BSA, LEA, and PLA, and the final Gd-BSA-LEA-Gd-PLA NPs were measured on a Bruker Tensor37 FTIR spectrometer using a Bruker Platinum ATR accessory equipped with a single reflection diamond crystal. Sample and reference spectra were recorded in the 4000–400 cm\(^{-1}\) spectral range with a resolution of 4 cm\(^{-1}\) and averaged over 128 scans. BSA, LEA, and the NP absorbance spectra were measured in phosphate buffer using phosphate buffer as reference. The PLA spectrum was measured as the pure dry compound (yellow pellet) using the clean diamond surface as reference. Bruker OPUS software was used to subtract a measured pure water vapor spectrum when small overlapping water vapor peaks occurred in the Amid region. Spectra were scaled in absorbance to compensate for different concentrations.

**Determination of Gd-LEA-BSA-Gd-PLA size**

**Transmission electron microscopy (TEM)** A suspension of NPs was centrifuged at 14,000 rpm for 10 min to obtain a pellet of approximately 1.0 mm\(^3\) volume, which was then fixed in 1% osmium tetroxide solution at 4°C overnight. Following dehydration in...
graded ethanols, 70–100% over two hours, the pellet was passed through two changes of acetone as an intermediate, infiltrated overnight with 1 : 1 Epon : acetone mixture, six hours with a fresh Epon : acetone mixture, and embedded in Epon and polymerized at 60°C for 48 hours. Semi-thin and ultrathin sections were cut, contrasted in lead and uranium salts, and then viewed on an electron microscope, at magnifications between ×5000 and ×40,000 (Zeiss EM 10 electron microscope). Images were printed, scanned for digitization, and employed in morphometric analysis of NPs diameter, size, number, internal structure, and interparticle behavior. Based on the knowledge of pellet weight and volume, and from counts of particle number in defined areas of the electron micrographs, the numbers of particles in one-milligram pellet could be obtained (data not shown).

**Scanning electron microscopy (SEM)** A 20 μl droplet of the NPs suspension was spread on a 12-mm glass coverslip and allowed to air-dry for two hours. The coverslip was then sputtered with a 5-nm coating of palladium gold. The specimen was then viewed in a scanning electron microscope with accelerating voltages of 1–5 kV, at magnifications from ×10,000 to ×100,000 (on a Zeiss DMS 952). Digitized images in TIFF format were obtained and employed in morphometric analysis of nanoparticle diameter, size distribution, shape, and interparticle behavior.

**Photon correlation spectroscopy (PCS)** To assess the sizes of the prepared NPs, a hydrodynamic light-scattering method was applied by using a Submicron Particle Sizer (Nicomp 380 DLS, California, USA). Measurements were usually carried out in purified millipore water. NPs’ concentrations were chosen to produce a measurement intensity of about 300 kHz. During measurements, the temperature was held constant at 23°C. The doubly derivatized NPs were measured for 90 min (three cycles, each with a 30 min run time). The Nicomp DLS instrument was calibrated using standard 92-nm latex beads (Duke Scientific, Fremont, USA) at three-month intervals. Nicomp intensity weight thresholds were used below 2%, and channel width was set to 10 μs, but, in AUTO mode, was 10–200 μs. Data were collected until convergence. Results are the average of three independent samples measured in triplicate. Zeta potential was measured in the same instrument.

**Correlative MRI and ultrastructural analysis of nanoparticles**

**Scanning electron microscopy (SEM)** Dehydration of the specimens was completed by three changes of absolute ethanol at 4°C, and then, the segments were transferred to acetone at 4°C for 30 min. The specimens were finally dried in a BAL-TEC CPD 030 critical point dryer, sputter-coated with gold palladium 5-nm thick, as described above, and viewed on a Zeiss DSM 952 (Gemini, Germany) scanning electron microscope. The images revealed the surface structure of the endothelial cells and the types of interactions between the nanoparticles and the cells.

**Transmission electron microscopy (TEM)** Following incubation in reduced ferricyanide, the vein segments were dehydrated in three changes of absolute ethanol at 4°C, passed through two changes of acetone (the first at 4°C, the second at room temperature) and embedded in Epon. Semi-thin sections were cut and viewed with light microscopy after staining with Toluidine Blue. Ultrathin sections were cut, contrasted with lead and uranyl salts (Reynolds 1963), and viewed on a Zeiss EM10 electron microscope (Germany). For all experiments carried out with human veins, we adhered to guidelines issued by the local Ethics Committee.

**Time course analyses of nanoparticle–vascular wall interactions**

- **Human model**
  - **Vena saphena magna, in vital condition**: the veins were obtained at surgery. Vein segments, approximately 1-cm long, were immediately cooled on ice in L15 culture medium and transported to the laboratory for further processing. The specimens were immersed in ice-cooled L15 containing 50 mg/ml Gd-LEA-BSA-Gd-PLA for five minutes. They were then warmed to 37°C in a water bath and incubated for 0, 5, 10, 15, 20, 30, 45, and 60 minutes. At the end of each incubation period, one vein segment was fixed by immersion in ice-cooled 2.5% glutaraldehyde in cacodylate buffer (0.1M, pH 7.4), for 18 hours. During this period, they were warmed briefly to room temperature, imaged with
MRI as described previously (Paschkunova-Martic et al. 2005), and fixation then continued at 4°C. Following fixation, the segments were rinsed repeatedly during 60 minutes in cacodylate buffer at 4°C, then immersed in 1% osmium tetroxide (in distilled water) for 24 hours at 4°C, and finally immersed in 70% ethanol for 24 hours at 4°C. Each vein segment was then cut into two pieces, one for SEM and the other for TEM.

- Vena saphena magna, aldehyde fixed: the veins were fixed in 2.5% glutaraldehyde in cacodylate buffer (pH 7.2, 0.1 M), at 4°C for up to six weeks.

In vitro magnetic resonance (MR) characterization

In vitro measurements of signal enhancement were correlated with different concentrations of nanoparticles. MR measurements were obtained from serially diluted Gd-LEA-BSA-Gd-PLA suspensions as described previously (Paschkunova-Martic et al. 2005). For the examination of small samples, a linearly polarized, small-loop receive coil was used (inner diameter 28 mm). The highest concentration of the nanoparticle suspension (100 mg/ml) used was diluted in steps of 1:10 using PBS as the solvent. The individual solutions were filled into 100-μl tubes (Eppendorf AG, Germany) and placed within the MRI receiver coil using a special sample holder.

MRI of vein segments was carried out as previously reported (Gong et al. 2016; Paschkunova-Martic et al. 2005). Briefly, 2D spin echo (SE) and 3D gradient echo (GE) sequences as normally used for contrast enhanced patient examinations were applied. Due to the small sample size, the sequences were modified to allow for a small field of view (FOV) in the range between 25 and 50 mm. The resulting sequence parameters were for (1) SE sequences: TR=300ms, TE=14ms, number of slices 9, slice thickness=2mm, slice distance factor=5%, acquisition matrix=256×256, number of excitations=2, and acquisition time=2 min 56 sec and (2) 3D GE-sequences: TR=32ms, TE=16ms, flip angle=50°, slab thickness=40 mm, number of slices=64, effective slice thickness=600 μm, acquisition matrix: 256×256, number of excitations=1, and acquisition time=8 min 44 sec.

Results

Preparation and physicochemical characterization of Gd-LEA-BSA-Gd-PLA nanoparticles

The physicochemical characteristics of the final contrast agent and its intermediate compounds are shown in Table 1. These compounds showed narrow size distributions in good agreement with three different methods for size determination (SEM (Figs. 3a and 3b), TEM (Fig. 4), and PCS (Fig. 5)) and possessed a negative surface charge (ζ-potential) between −26 and −30 mV. Detailed calculations of the maximum possible number of Gd atoms tessellating a single NP are shown in Supplements I and II.

When comparing the NP ATR-FTIR spectrum with the spectrum of PLA, the contribution of PLA in the NP spectrum is evident. The most prominent bands of BSA are in the Amid I region at 1650 cm⁻¹ and in the Amid II region at 1548 cm⁻¹ (Fig. 2). The spectrum of LEA additional contains strong bands resulting from galactose and arabinose fractions in the 1000 cm⁻¹ region. In the spectrum of the NPs, only the Amid II bands can be clearly assigned to protein absorption of both, BSA and/or LEA. On the one hand, Amid I is overlapping with the δ-H₂O band of the solvent and can thus disappear in the

| Compound                  | BSA-/BSA-Gd (% w/v) | Aver. Size [nm]# | ζ-potential [mV]* | cGd** [mol/l] | cBSA/LEA** [mol/l] | Molar ratio cGd/c Protein |
|---------------------------|---------------------|------------------|-------------------|---------------|---------------------|--------------------------|
| Plain BSA-PLA NPs         | 20                  | 188              | -26               | -             | 12.3e-6             | -                        |
| BSA-Gd-PLA                | 20                  | 213              | -30               | 6.30e-4       | 2.91e-5             | 21.7                     |
| BSA-Gd-PLA                | 2                   | 55               | -30               | 6.30e-4       | 2.91e-5             | 21.7                     |
| Gd-LEA-BSA-Gd-PLA         | 2                   | 65               | -26               | 1.11e-4       | 6.00e-6             | 18.5                     |

# SEM measurements; *PCS refractive index (RI=1.333), viscosity=0.933 cP; **values determined before BSA-PLA encapsulation

Table 1 Physicochemical characteristics of Gd-LEA-BSA-Gd-PLA nanoparticles and their intermediate compounds
absorbance spectrum of proteins at high concentrations of other components (here PLA). This fact is well known for measurements of proteins in aqueous solutions. On the other hand, the sugar absorption overlaps with the PLA absorption in a large area. Nevertheless, there is a small band visible at 994 cm⁻¹ in the NP-spectrum that can be assigned to a band only present in the spectrum of LEA and thus shows LEA content in the spectrum of the NPs (Fig. 2).

Correlative MRI and ultrastructural analysis of nanoparticles

The relative signal enhancement of Gd-LEA-BSA-Gd-PLA compared to plain, control PLA-BSA NPs is shown in Fig. 6. A steep signal increase by increasing concentrations is observed with Gd-LEA-BSA-Gd-PLA, whereas no signal increase is seen with plain BSA-PLA NPs.

Fig. 2 ATR-FTIR spectra of free BSA, LEA, and PLA and the final Gd-BSA-LEA-Gd-PLA NPs. The gray line represents pure PLA as provided by the manufacturer, the green and blue lines represent BSA and LEA alone respectively, and the brown line represents the spectrum of final Gd-BSA-LEA-Gd-PLA. The peaks in each line reflect the main functional groups.

Fig. 3 a Scanning electron microscope image of Gd-LEA-BSA-Gd-PLA nanoparticles in a batch of 55-nm spheroid monodisperse population. Magnification is ×50,000 and the scale bar 500 nm. b Scanning electron microscope image of Gd-LEA-BSA-Gd-PLA nanoparticles in a batch of 75-nm spheroid monodisperse population. Magnification is ×100,000 and the scale bar 100 nm.
Different fractions of two different batches of Gd-LEA-BSA-Gd-PLA are shown in Fig. 7. Interbatch preparations possessed a similar high signal increase at MRI.

In vitro MRI studies

In vitro MRI of human vena saphena magna: compared to different control substances and intermediate compounds, only Gd-LEA-BSA-Gd-PLA was taken up by the endothelial cells, leading to a strong signal at MRI (Fig. 8). These findings were confirmed by TEM (Fig. 9).

Discussion

In this study, we synthesized Gd-LEA-BSA-Gd-PLA, a doubly derivatized polylactic acid-bovine serum albumin nanoparticle, stabilized by covalent crosslinking with glutaraldehyde. Modifications, including the introduction of two surface layers of targeting groups (LEA) and signal-emitting moieties (DTPA-Gd chelates), resulted in high Gd payloads that enabled specific labeling of the endothelial cells of vena saphena magna and a strong signal enhancement at apical EC surface in MRI, while preserving binding affinity and specificity of LEA.

We applied Gd as a well-established signal enhancer in MRI, which provided a strong signal on T1-weighted MR sequences and selected DTPA as a chelator, although we were aware of the significantly greater stability of DOTA and other macrocyclic compounds. Moreover, this further development of albumin-based nanoparticles retained DTPA from our earlier proof-of-principle work, in which the first synthesis and standardization of human albumin-PLA (HSA-PLA) NPs for use in MRI was reported (Stollenwerk et al. 2010). The relative signal enhancement in dilution series with
Gd-LEA-BSA-Gd-PLA showed a steep increase, with a maximum at about 1200%. Interbatch populations, as well as within the same fraction of NPs, showed a similar increase. Nevertheless, difference in the signal enhancements caused by the collected second fractions (fractions 2 after FPLC) of two separate batches was observed. This fact could be explained by variable loading efficiency with Gd and/or difference in the rotational correlation times of the final constructs during MRI measurements. The specific binding of the new compound toward EC was examined and proved in human *vena saphena magna* tissue.

In the preparation of nanoscale MRI contrast agents, particle size, and size distribution are important parameters that determine their fate in *in vivo* studies. In the current study, small-size Gd-LEA-BSA-Gd-PLA was produced by reducing surfactant concentration to 2% w/v and homogenizing the reaction emulsion for 3 min at 24,000 rpm. BSA served as a surfactant, which was attached mainly through hydrophobic interactions to the negatively charged PLA nanoparticles and additionally reinforced by glutaraldehyde crosslinking. As proven by Montisci et al., the release of BSA was significantly lowered after glutaraldehyde activation, suggesting that the crosslinking of BSA occurred at least at the particle’s surface. According to our estimations, there are BSA molecules underneath this surface, as well as on it (see Supplements I and II).

Montisci et al. reported that glutaraldehyde might help to create and stabilize a firmly anchored layer of BSA at the surface of micron-range PLA microspheres, which resists detachment by surfactants.

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**Fig. 6** Relative signal enhancement to concentration curve of Gd-LEA-BSA-Gd-PLA compared to plain, control BSA-PLA NPs

**Fig. 7** Relative signal enhancement to concentration curve of different fractions (1, 2, and n (pellet)) of two different batches of Gd-LEA-BSA-Gd-PLA NPs
Polymerized glutaraldehyde molecules, resulting at neutral or basic pH from an aldol condensation, react with the amino groups from BSA and form an imine bond, which is stabilized by the presence of a conjugated ethylene bond (Paschkunova-Martic et al. 2005). Free remaining aldehyde groups, in turn, allow the propagation of the crosslinking reaction or the fixation of exogenous molecules, such as lectins. As shown in this study, a sufficient attachment of LEA was achieved to demonstrate specific binding of the Gd-LEA-BSA-Gd-PLA to ECs in blood vessel walls.

**Fig. 8** In vitro MRI of human vena saphena magna specimens. Probe **A**: PBS (negative control); probe **B**: Gd-LEA-BSA-Gd-PLA, 0.11 mM Gd; probe **C**: 0.1 mM gadopentate (gadopentetate dimeglumine (“Magnevist”); positive control); probe **D**: plain BSA-Gd-PLA, 0.063 mM. Note the Gd-LEA-BSA-Gd-PLA uptake of the endothelial cells (**B**: white line; veins’ segments were everted so the endothelial layer faces outwards) and no uptake in probes **C** and **D**.

**Fig. 9** Human vena saphena magna incubated (in fixed condition) with Gd-LEA-BSA-Gd-PLA (**image a**). Transmission electron microscopy confirmed binding of Gd-LEA-BSA-Gd-PLA to endothelial cells. Gd-LEA-BSA-Gd-PLA formed a dark layer (arrows) on the luminal surface of the endothelium in **a**, but not in **b** (control specimen with fixation only).
We chose BSA as a surfactant, as it is a naturally abundant protein in the living organism and can be easily modified with DTPA-Gd chelates. BSA is cheap and plentiful and can be replaced by HSA (human serum albumin) for future clinical applications. It can be obtained by simple protocols from blood, or can be prepared as a recombinant protein. The industrial handling of this protein is well understood, its binding sites for drugs are especially well studied, and it has been used as a carrier of Gd chelates for use with MRI (Daldrup-Link and Brasch 2003). PLA was used as a starting material for the NP production, as it is a biodegradable biopolymer that is expected to be metabolized soon after the MRI examination into smaller lactic acid subunits for renal excretion.

Ideally, a diagnostic contrast agent should be eliminated completely and relatively soon after completion of the imaging examination (Beckett et al. 2015; Lauffer et al. 1998). In this respect, further investigations with the novel Gd-LEA-BSA-Gd-PLA with regard to in vivo studies and pharmacokinetic and elimination are needed. Both BSA alone and the BSA-PLA combination have been used previously to create microspheres (Boury et al. 1997; Jiang and Schwendeman 2001; Müller et al. 1996). The novel aspect of the present study lies in the unique chemical structure of the NPs, which comprises the inner hydrophobic core (PLA chains) and modified hydrophilic double layers of the shell that contains BSA-Gd and LEA-Gd chelates (signal-emitting and tissue-targeting groups) on a single NP. This fact allows possible further loading of the constructs with other biologically active compounds with poor water solubility (e.g., anti-tumor metal complexes) and the development of novel theranostic probes (manuscript in preparation).

The total amount of Gd in MRI CAs is a major prerequisite for achieving a good signal enhancement. According to our calculations, the possible maximum number of BSA molecules on a PLA NP is 625 (see Supplement II). Presumably, an average of 20 Gd-DTPA chelates bind to one BSA and about 0.03 LEA could be crosslinked on it. Consequently, over 1200 Gd atoms will be present throughout a single Gd-LEA-BSA-Gd-PLA. As this is a very high number, future investigations should focus on optimizing the NP synthesis, particularly by inclusion of real-time analytical steps. The high Gd payload we achieved, or, generally, the transmetallation from DTPA chelates, represents a potential Gd metal toxicity. With regard to NP toxicity, we expect no cytotoxicity or immunogenicity, as previously reported by us with PLA-human serum albumin NPs with just one Gd layer (Stollenwerk et al. 2010). Moreover, these materials provide a platform for further development of clinically relevant macromolecular contrast agents and can be up-scaled for industrial fabrication.

NP size is a crucial parameter in the evaluation of the magnetic resonance properties of a novel macromolecular agent. As previously described, the optimal size should not exceed the blood capillary diameter or damage the blood vessels (Gong et al. 2016; Paschkunova-Martic et al. 2005). We used three methods in tandem because their advantages are complementary. The PCS models the size distributions of large nanoparticle populations and demonstrates the presence of aggregates, whereas TEM and SEM allow direct measurement of a few hundred nanoparticles but underestimate the aggregates. TEM in addition provides valuable insight into NP integrity.

Gd-LEA-BSA-Gd-PLA showed diameters with a narrow size range, as well as evidence that this compound can be readily standardized. Furthermore, we demonstrated that Gd-LEA-BSA-Gd-PLA, a small (55–75 nm) NP, can generate strong MR signals in the vessel wall. NP size was found to be a major determinant of biological response. An optimal size must be smaller than 100-nm diameter, as earlier reported (Paschkunova-Martic et al. 2005; Stollenwerk et al. 2010). The surface charge of the Gd-LEA-BSA-Gd-PLA, measured as a zeta potential, is sufficient to repel neighboring nanoparticles, and thus, hold the suspension in the form of individual particles and lies between -26 and -30 mV. In this case, a suspension of charged individual particles retains the characteristic features of the nanosize scale, for example, the enormous surface area. In contrast, particles with little charge may clump into aggregates, which have the same total mass as the nanoparticle suspension but have a strongly reduced surface area. Such aggregates are not desirable, as they cannot be provided with targeting moieties, and they would likely enter into interactions with living tissues different from those observed for individual nanoparticles.

The use of the tomato lectin LEA was designed to bind to normophysiological ECs at blood vessels. The control BSA-PLA NPs generated no signals in the fixed *v. saphe. magna*, while Gd-LEA-BSA-Gd-PLA did bind very well, indicating carbohydrate specificity. Specific binding to the ECs was demonstrated in vitro at MRI and correlated to microscopic analyses. In this
regard, strong MR signals in the vessel wall in fixed human vein segments have been shown. However, further studies are needed to determine the optimal NP constitution. Ongoing work is focused on replacing tomato lectin with a tumor-specific ligand and DTPA with cyclic ligands to optimize synthesis procedures and avoid potential safety risks.

**Conclusion**

In this work, we have synthesized and evaluated the feasibility of Gd-LEA-BSA-Gd-PLA nanoparticles. In vitro MRI, confirmed by TEM and SEM analyses, certified the binding affinity and specificity of Gd-LEA-BSA-Gd-PLA to EC of human veins and a strong persistent enhancement at the EC apical surface. The stabilized core–shell structure of Gd-LEA-BSA-Gd-PLA might allow for further encapsulation of lipophilic drugs or for the attachment of different targeting molecules, such as antibodies.

The potential of Gd-LEA-BSA-Gd-PLA NPs as an EC-targeted contrast agent should be examined further in vivo for possible use for visualization of areas with vessels of varying permeability (e.g., tumors). They also can help to define ischemia and reperfusion after treatment, for example, in cerebral or myocardial infarction (Leigh et al. 2018; Naresh et al. 2011).

Nowadays, highly stable paramagnetic complexes of Gd(III) are extensively used in MRI procedures (Zhou and Lu 2013). Anytime, there is the need for visualizing small tumors or abnormalities before the physical manifestation of a disease. Currently, the relaxivity of MR contrast agents, especially for molecular imaging applications, should be very high to deal with the low sensitivity of MRI (Terreno et al. 2010). PLA-BSA-LEA NPs bearing multiple contrast groups may provide signal amplification and overcome this limitation. In principle, they can deliver both contrast medium and drug, allowing monitoring of biodistribution and therapeutic activity simultaneously (theranostics) (Debbage and Jaschke 2008; Terreno et al. 2010).

By combining the present approach with existing strategies, multifunctional PLA-BSA carriers might be designed as multi-modal particles (e.g., PET-MR or OI-MR) for simultaneous imaging with different imaging techniques as well as targeted contrast agents for site-specific imaging. Moreover, the prepared PLA-BSA-LEA NPs may act as transport vesicles for cytotoxic drugs with poor water solubility. Thus, new and refined theranostic insights might become reality.

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**Data Availability** All data supporting the findings of the article is available in the local institutional repository of the Department of Inorganic Chemistry, University of Vienna and will be provided on reasonable request by the corresponding author.

**Code availability** Not applicable.

**Compliance with ethical standards**

**Ethics approval** For all experiments carried out with human veins, we adhered to guidelines issued by the local Austrian Ethics Committee.

**Consent to participate** Not applicable

**Consent for publication** Not applicable

**Conflict of interest** The authors declare that they have no conflict of interest.

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