Stabilization of Nanoparticles Synthesized by Miniemulsion Polymerization Using "Green" Amino-Acid Based Surfactants

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Summary: In this study biocompatible amino-acid based surfactants were used for the stabilization of polystyrene (PS), poly(L-lactide) (PLLA) and polyphosphate (PP) nanoparticles (NPs). The formation of the nanoparticles was achieved either by radical polymerization in miniemulsion for polystyrene nanoparticles or by using a combination of solvent evaporation and miniemulsion approach for poly(L-lactide) or polyphosphate nanoparticles. The influence of the three different amino-acid based surfactants, which were used with different amounts, on the NPs' stability, size, size distribution, zeta potential, morphology and surface tension is discussed. Cell experiments with HeLa cells were performed for the determination of the toxicity. For comparison, sodium dodecyl sulfate (SDS) stabilized NPs were synthesized, and all results obtained from the amino-acid based surfactant stabilized NPs were compared and discussed with the SDS stabilized NPs.

Keywords: miniemulsion polymerization; polystyrene; stabilization; surfactants

Introduction

The use of different stabilizing molecules allows the modification of the nanoparticles surface even in the formation of nanoparticles with different types of surfactants. The most commonly used surfactant is anionic sodium dodecyl sulfate (SDS). Despite the fact that SDS is not carcinogenic when applied directly to the skin or when consumed, it is known that SDS deactivates enzymes and can denature proteins.[1] As a result, this surfactant cannot be used for enzyme triggered release mechanisms in drug delivery systems. Therefore the need for replacement of SDS through stabilizing molecules with biocompatible and biodegradable properties, with good stabilization behavior and with a reduced toxicity, is self-evident.

Amino-acid based surfactants are a good alternative to classical ionic surfactants because of their biocompatibility and biodegradation. Due to the fact that the surfactant remains bound to the surface of the final particle system, it is necessary to achieve minimal toxicity. Data about the structure, preparation and properties of amino-acid based surfactants are reported by Infante *et al.*[2] These surface active compounds consist of an amino-acid residue polar head group which enhances the water solubility whereas the hydrophobic tail guarantees the micellization.[3] Rosenbauer *et al.* studied the synthesis of (meth) acrylate water-borne latexes using amino-acid based surfactants and their effects on film formation.[4] The physicochemical properties such as hydrophilic-lipophilic balance values, minimum surface tension, critical micelle concentration (cmc) values and the physiological and environmental properties of the aforementioned amino-

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acid based surfactants are reported and compared with SDS in a detailed article of Hampshire, a subsidiary of the Dow Chemical Company.[5]

Due to the hydrophilic and hydrophobic moieties, the amino-acid based surfactants can effectively stabilize nanoparticle systems. Using the miniemulsion process, a large droplet surface area is created by applying high shear forces. The miniemulsion system is a heterophase system consisting of a continuous oil-phase and a water phase. Under ideal conditions the sizes of the final particles are the same as the sizes of the initial miniemulsion droplets. Carefully prepared miniemulsions result in a monodisperse nanoparticle size distribution with nanoparticles in the size range of 50–500 nm that can be controlled by variation of the type and concentration of surfactant and monomer.[6] The distribution of the miniemulsion droplets mainly depends on the amount and type of hydrophobic agent used in the system and the high shearing parameters.[7]

The goal of this study was to synthesize polystyrene, poly(L-lactide) and polyphosphate nanoparticles stabilized with different amino-acid based surfactants (sodium lauroyl sarcosinate (SLS), sodium lauroyl glutamate (SLG), sodium myristoyl sarcosinate (SMS)) using a miniemulsion protocol. For comparison, nanoparticles with sodium dodecyl sulfate (SDS) were also synthesized. The influence on the stability, size, size distribution, morphology and surface tension by using different amounts of the surfactants SLS, SLG and SMS was investigated, compared with SDS stabilized NPs and discussed. The toxicity of these NPs was evaluated by a MTS assay.

Experimental Part

Materials

Styrene (Merck) was purified by distillation under reduced pressure before use. All the other reagents and solvents were used as received. The oil-soluble initiator 2,2′-azobis(2-methylbutyronitrile) (V59) from Wako Chemicals, Japan, was used as initiator. Hexadecane was purchased from Aldrich. Biomer®L9000 was kindly supplied by Biomer, Germany (M_n: 66,500 g/mol, M_w: 145,000 g/mol determined by GPC in chloroform). The HPLC-grade organic solvent chloroform was purchased from Merck. Sodium dodecyl sulfate (SDS) was purchased from Merck, sodium myristoyl sarcosinate (SMS) and sodium lauroyl glutamate (SLG) were kindly provided by Schill and Seilacher, and sodium lauroyl sarcosinate (SLS) was purchased from AppliChem. The polyphosphate polymer (PP) was synthesized according to method previously described in the literature,[8] being previously hydrogenated before the use with a Pd/C catalyst (5% Pd/C) purchased from Aldrich. Demineralized water was used in all experiments.

The chemical structures of the polymers and all surfactants are depicted in Figure 1.

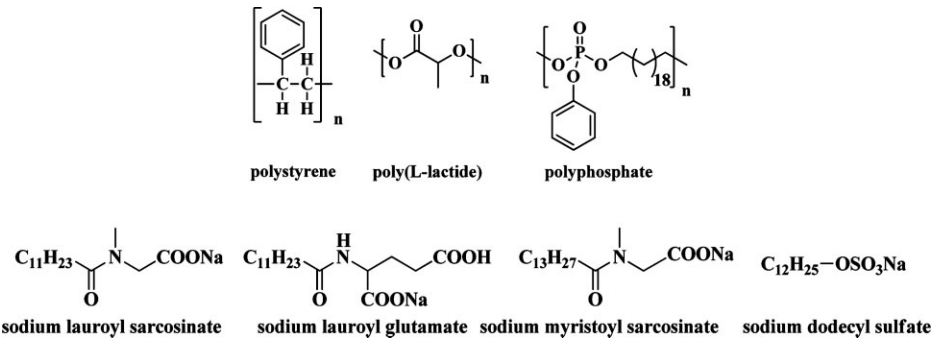

Figure 1.
Chemical structures of the polymers and the used surfactants.

Synthesis of Polystyrene Based Nanoparticles (PS-NPs)

Polystyrene-based nanoparticles were synthesized via miniemulsion polymerization according to the previously described procedure.[9] Briefly, a total monomer amount of 6 g styrene, 250 mg hexadecane, and 100 mg V59 as initiator were mixed and added to the aqueous phase (24 g water and different amounts of SLS, SLG, SMS and SDS). After 1 h of stirring at 1000 rpm, the mixture was homogenized by ultrasonication for 120 s at 90% intensity (Branson sonifier W450 Digital, 1/2″ tip) at 0 °C in order to prevent polymerization of the monomer(s). Polymerization was carried out at 72 °C under stirring for 12 h. For the characterization all polymeric nanoparticles were dialyzed for 48 h (MWCO: 12,000 g/mol) in order to remove the residual surfactant – if any. The formulation process is schematically shown in Figure 2.

Synthesis of Poly(L-Lactide) and Polyphosphate Based Nanoparticles (PLLA-NPs or PP-NPs)

The synthesis of the PLLA-NPs was achieved by the combination of the solvent evaporation and miniemulsion method. Briefly, 300 mg PLLA was dissolved in 10 g of chloroform at 40 °C. Afterwards the aqueous phase consisting of 24 g water and different amounts of surfactant (SLS, SLG, SMS and SDS) was added to the chloroform phase. For the PP based NPs, 15 mg of polyphosphate was dissolved in 1.25 g of chloroform at 25 °C. 5 g water and different amounts of surfactant (SLS, SLG, SMS and SDS) were added to the chloroform phase in the same proportion used for the preparation of the PLLA-NPs. The solution was stirred at 600 rpm over 1 h at 25 °C. Afterwards, the miniemulsion was prepared by ultrasonication for 180 s at 70% amplitude in a pulse regime (30 s sonication, 10 s pause) using a Branson 450 W sonifier and a 1/4″ tip. The sonication was performed under ice cooling in order to prevent evaporation of the solvent. The obtained miniemulsion was transferred into a 50 mL reaction flask with a large size neck and kept overnight at 40 °C for a complete evaporation of the chloroform. The polymeric NPs were cleaned by dialyzation (MWCO: 12,000 g/mol) over 48 h in order to remove the residual surfactant – if any. The formulation process is schematically shown in Figure 3.

MTS Assay for Determination of Cell Viability

HeLa cells were kept in Dulbecco's Modified Eagle Medium (DMEM) without Phenolred supplemented with 10 vol% fetal calf serum (FCS), 100 units penicillin and 1 vol% GlutaMAX™ (all from

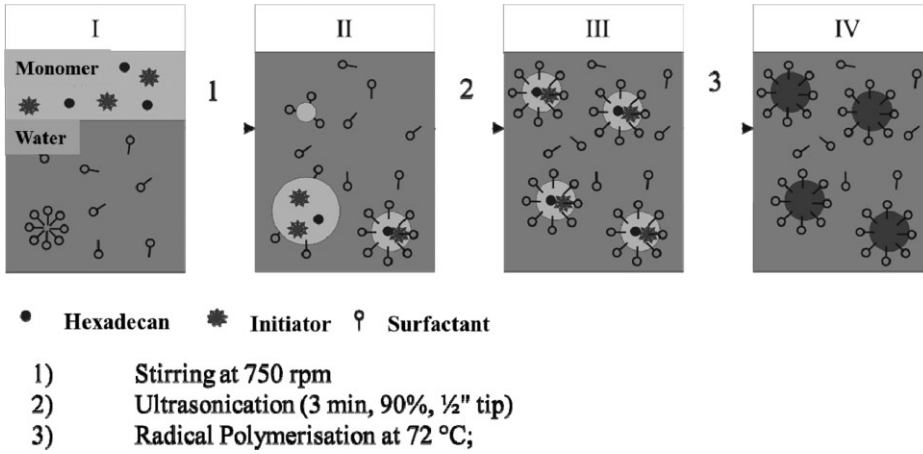

Figure 2.
Formulation process for radical polymerization in miniemulsion.

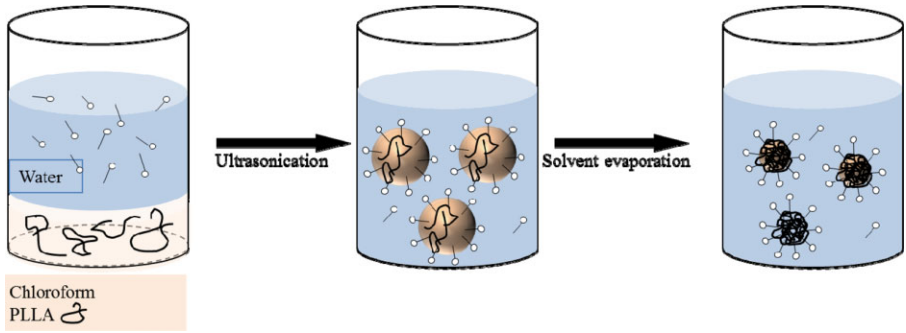

Figure 3.
Formulation process for solvent evaporation combined with the miniemulsion technique.

Invitrogen, Germany). Cells were grown in a humidified incubator at 37 °C and 5% CO_2. A MTS assay was performed to show if the synthesized nanoparticles have a toxic influence on HeLa cells. Therefore cells were detached using 0.5% trypsin (Gibco, Germany) and seeded out in 96 well plates (Becton Dickinson, USA) at a density of 0.8×10^5 cells/well. After re-adhesion over night cells were incubated with each nanoparticle at a concentration of 75, 150, 45, 600, 750 and 1000 μg/mL for 24 h in a humidified incubation at 37 °C and 5% CO_2. After incubation time the MTS assay was done in medium following the manufacturer's protocol of CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega, USA). MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) is reduced NADH-dependent by cells to a colored formazan product which is soluble in culture medium. The concentration of formazan (directly proportional to the number of live cells) is photometrically detectable as absorption at 490 nm. The absorption signal was obtained by using a Plate Reader Infinite M1000 (Tecan, Germany). Cell viability was determined by using the method previously reported in literature.[10][11]

Analytical Methods

The average size and the size distribution of the NPs by means of dynamic light scattering (DLS) were measured with diluted dispersions (40 μL sample was diluted in 1 mL water) on a PSS Nicomp Particle Sizer 380 (Nicomp Particle Sizing Systems, USA) equipped with a detector at 90° scattering mode at 20 °C. The zeta potential of the NPs was measured in 10^{-3} M potassium chloride solution with a Zeta Nanosizer (Malvern Instruments, U.K.) at 20 °C. Scanning electron microscopy (SEM) images were recorded by using a field emission microscope (LEO (Zeiss) 1530 Gemini, Oberkochen, Germany) working at an accelerating voltage of 170 V. Generally, the samples were prepared by diluting the NP dispersion in demineralized water to about 0.01% solid content; then one droplet of the sample was placed onto silica wafers and dried under ambient conditions over night. Surface tension measurements were performed by the DuNouy ring method with a DCAT21 tensiometer (Dataphysics) at 25 °C.

Results and Discussion

To test the stabilization behavior of the three different amino-acid based surfactants used in three different concentrations, the synthesis of PS, PLLA and PP nanoparticles by a miniemulsion polymerization was performed. As a control, SDS as commonly utilized surfactant was used. Polystyrene was chosen as a model system due to its inert and well investigated polymeric properties. Furthermore, the

direct polymerization process in miniemulsion was performed in a controlled way. In contrast to stabilization of a miniemulsion during and after radical polymerization, two polyester systems were investigated due to their biodegradability and biocompatibility. PLLA is the typical polyester in the biomedical, pharmaceutical and drug delivery field.[12] Polyphosphates came into focus rather recently as highly promising polyesters that combine degradability and polyfunctionality with several synthetic advantages over conventional polyesters.[13]

The nanoparticles were synthesized in a direct (oil-in-water) miniemulsion system through either radical polymerization (in the case of PS) or the solvent evaporation technique (in the case of PLLA and PP). For the stabilization different types (SLS, SLG, SMS) and different amounts of surfactant (72 mg, 108 mg or 144 mg in 24 g water) were used. SDS served as control. After the synthesis and purification (dialysis) of the nanoparticles, they were analyzed in terms of average size, size distribution, morphology, zeta potential and surface tension. The obtained results are summarized in Table 1, Table 2 and in Figure 4. For morphology the SEM studies are depicted in Figure 5.

From the data in Table 1 it can be seen that all investigated surfactants and polymers can be used to produce stable nanoparticle dispersions via a miniemulsion protocol. Further, the diameters of the different nanoparticles decrease with increasing surfactant content in all cases, as expected. For all PS-NPs, independently of the surfactant, narrow size distributions of the nanoparticle dispersions were measured

Table 1.
Diameter, standard deviation and zeta potential of PS, PLLA and PP nanoparticles synthesized via the miniemulsion process.

Sample	Diameter, nm	Standard deviation, %	Zeta potential, mV (pH 7)
PS-SDS-72 (control)	100	11	−55
PS-SLS-72	100	11	−52
PS-SLS-108	90	10	−55
PS-SLS-144	85	9	−53
PS-SLG-72	95	3	−51
PS-SLG-108	90	16	−47
PS-SLG-144	85	17	−50
PS-SMS-72	92	17	−59
PS-SMS-108	85	11	−54
PS-SMS-144	80	12	−57
PLLA-SDS-72 (control)	90	30	−49
PLLA-SLS-72	130	26	−58
PLLA-SLS-108	120	30	−52
PLLA-SLS-144	100	30	−58
PLLA-SLG-72	190	28	−57
PLLA-SLG-108	180	28	−65
PLLA-SLG-144	140	30	−47
PLLA-SMS-72	160	18	−50
PLLA-SMS-108	140	30	−57
PLLA-SMS-144	125	30	−55
PP-SDS-72 (control)	130	13	−48
PP-SLS-72	135	24	−53
PP-SLS-108	120	20	−49
PP-SLS-144	115	20	−47
PP-SLG-72	190	23	−53
PP-SLG-108	200	20	−59
PP-SLG-144	180	27	−59
PP-SMS-72	170	29	−56
PP-SMS-108	150	17	−52
PP-SMS-144	130	19	−60

Table 2.
Surface tension of PS and PLLA nanoparticles synthesized via the miniemulsion process. The continuous phase consists of 24 g water and different amounts of SLS, SLG, SMS and SDS (72 mg, 108 mg and 144 mg in 24 g water).

Sample	Surface tension, mN per m PS-NPs		Surface tension, mN per m PLLA-NPs
SLS-72	67.5 (+/−0.4)		29.7 (+/−0.4)
SLS-108	66.5 (+/−0.4)	↓	29.0 (+/−0.4)
SLS-144	64.5 (+/−0.2)		27.4 (+/−1.2)
SLG-72	69.2 (+/−1.0)		26.5 (+/−0.2)
SLG-108	68.6 (+/−0.4)	↓	24.8 (+/−0.4)
SLG-144	67.9 (+/−0.5)		24.2 (+/−0.8)
SMS-72	69.6 (+/−0.4)		30.5 (+/−0.4)
SMS-108	68.2 (+/−0.2)	↓	30.0 (+/−0.4)
SMS-144	66.1 (+/−0.7)		28.5 (+/−0.7)

(lower than 17%). Furthermore, it could be seen for all PS nanoparticles, stabilized with an amino-acid based surfactant that they are in all cases slightly smaller in size compared to the control sample synthesized with SDS. For the PLLA-NPs and PP-NPs which were synthesized by the solvent evaporation/miniemulsion method the size distribution is broader compared to the PS-NPs and compared to the control samples (PLLA and PP nanoparticles stabilized with SDS). Looking at the size, it can be seen that amino-acid based surfactant stabilized PLLA-NPs and PP-NPs are bigger compared to the SDS stabilized PLLA-NPs and PP-NPs. However, with increasing surfactant content, the diameter of resulting nanoparticles decreases for PLLA-NPs and PP-NPs, respectively.

From the zeta potential measurements it could be seen that with zeta potentials of about −50 mV for all synthesized nanoparticles a very good stability was observed.

As expected, with higher surfactant amounts lower surface tension values were obtained. This behavior was observed for both nanoparticles, independently of the surfactant. Furthermore, lower surface tension values were measured for PLLA-NPs compared to the surface tension values of the corresponding PS-NPs. This means that in the formulation process lower amounts of surfactant are necessary for the stabilization of droplets and later on for the nanoparticles in the case of PLLA. The surface tension is lower due to the fact that there are more surfactant molecules in the water phase.

As seen from Figure 4, for the PS-NPs, the surface tension for SDS stabilized nanoparticles is the same as that for amino-acid based surfactants, which indicates that equal amounts are necessary for

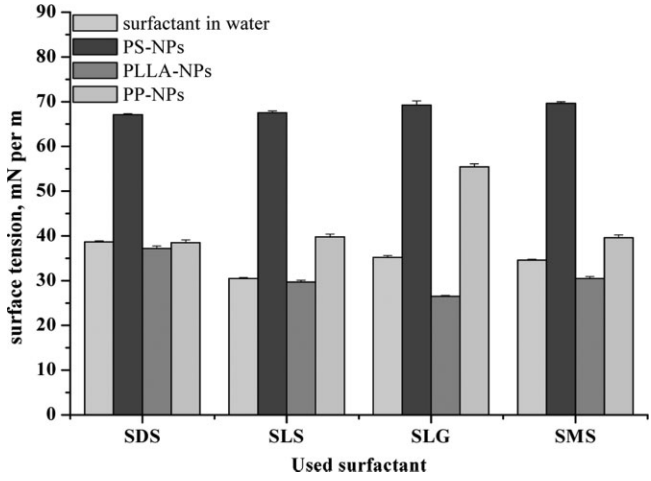

Figure 4.
Surface tensions measured for the surfactant/water solution and for PS-NPs, PLLA-NPs and PLLA-NPs. All nanoparticles were stabilized with 72 mg in 24 g water surfactant solution.

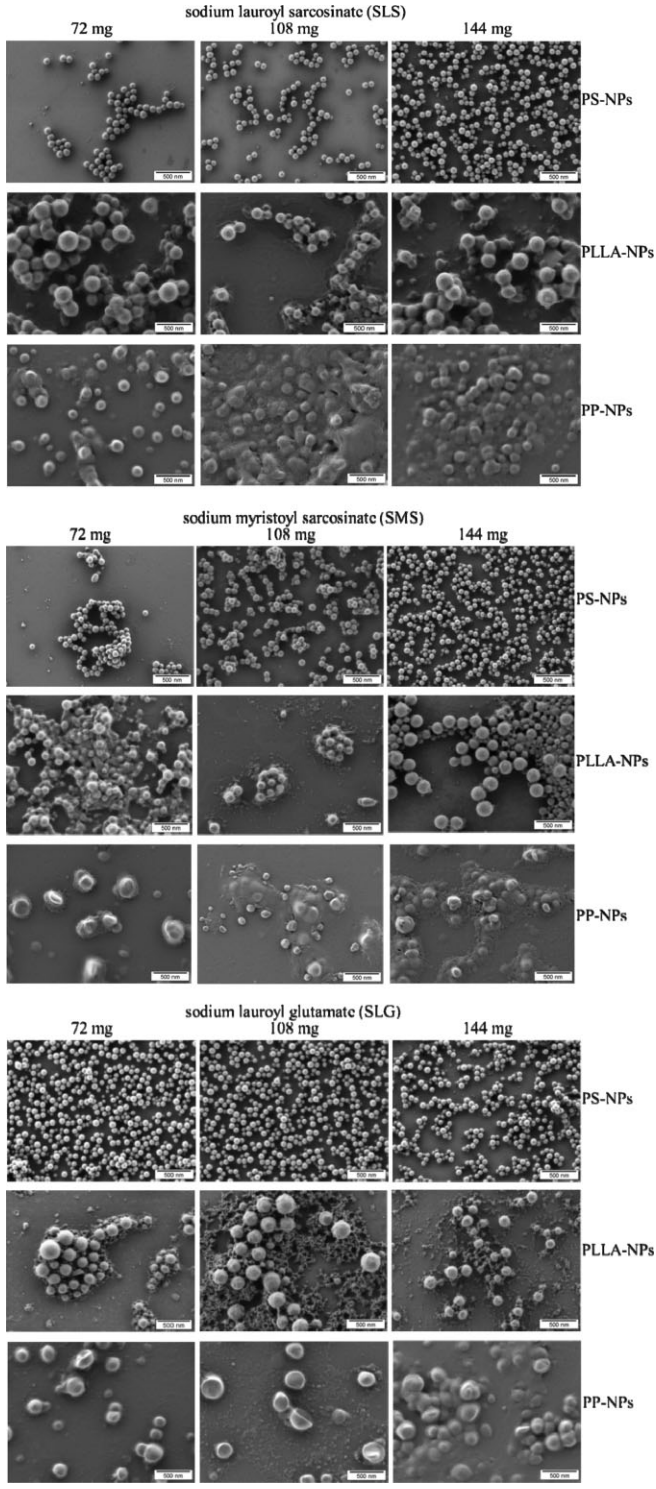

Figure 5.
SEM images of PS, PLLA and PP nanoparticles stabilized with different amounts of SLS, SLG and SMS.

the stabilization. For the PLLA-NPs, the surface tension for the SDS stabilized nanoparticles is slightly higher compared to the amino acid based surfactant stabilized nanoparticles indicating that less of the amino-acid based surfactant is necessary to stabilize the droplets and then the nanoparticles compared to SDS. This was observed for all amino-acid based surfactants. Looking at the PP-NPs, it can be seen that higher amounts of amino-acid based surfactants are needed for the stabilization compared to SDS stabilized NPs. Especially for SLG stabilized PP-NPs, a surface tension value increase of about 15 mN/m was measured.

From the measurements of surfactant in water (72 mg in 24 g water) it can be seen that the surface tensions are slightly lower for SLS, SLG and SMS compared to SDS. From the comparison of the surface tension of surfactant/water solution to the dispersions, nearly the same results were observed for PLLA. Despite having used the same procedure (combination of solvent evaporation and miniemulsion technique) for PP-NPs, higher amounts of surfactant are necessary compared to PLLA-NPs (comparing the surfactant/water solution to the dispersion).

The morphology of the NPs was visualized via SEM (Figure 5). The images show spherical PS-NPs with a monodisperse size distribution independently of the used surfactant. The same results were obtained for the PLLA-NPs. Due to the low melting point (of approximately 50 °C) of the PP [8], the particles were coated with a layer of carbon before analyzed by SEM. Despite the carbon sputtering in some cases of the PP-NPs a film formation behavior was observed because of the low melting point. The mean average size is slightly smaller than the one detected by dynamic light scattering due to the drying effects in the high vacuum environment of the SEM.

MTS Assay for Determination of Cell Viability

In order to show the advantage of amino acid-based surfactants compared to SDS and to ensure biocompatibility of nanoparticles stabilized with these surfactants, MTS assays were performed on samples based on each of the surfactants in comparison with SDS-stabilized nanoparticles. The attention was turned to PLLA and PP nanoparticles, because these are the biologically relevant particles of this work regarding to their biodegradability. Figure 6 shows the influence of the nanoparticles (prepared with 72 mg surfactant) on HeLa cells after 24 h incubation time. PLLA nanoparticles stabilized with SLS or SLG show no toxic effect at any concentration even at the highest of 1000 μg/mL due to cell viability of far beyond 80% relative to negative control. Significant cell death occurred with SMS-stabilized nanoparticles at a concentration of 600 μg/mL. But this is far away of any concentration administered in biomedical applications. Below 600 μg/mL there is also no toxic influence of SMS-stabilized particles. In the case of the PP particles, the toxicity was even lower, which identifies these nanoparticles as high potential candidates for further evaluations.

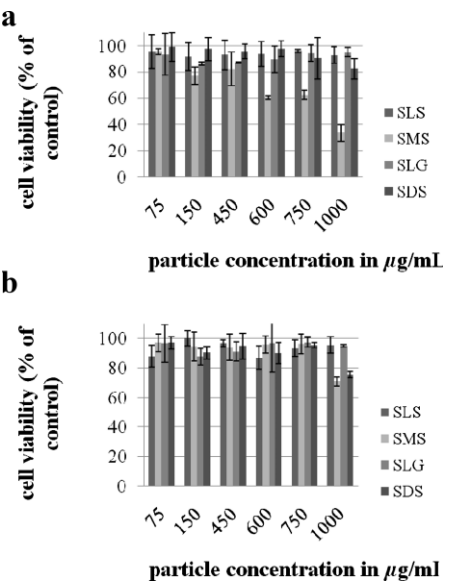

Figure 6.
MTS assay of a) PLLA and b) PP nanoparticles stabilized with the amino acid-based surfactants SLS, SMS and SLG in comparison with SDS-stabilized nanoparticles (surfactant amount 72 mg) in HeLa after 24 h.

Conclusion

Amino-acid based surfactants were used in the efficient formation of PS, PLLA or PP nanoparticles via the miniemulsion approach. These "green" surfactants are environmentally friendly, biodegradable and have on one hand proven to produce nontoxic nanoparticles for different polymer classes with PS being the model particle and on the other hand to serve as a surface active compound for the stabilization of different nanoparticles. Furthermore, PLLA and PP nanoparticles were synthesized via solvent evaporation miniemulsion strategy as model particles for biocompatible systems proving the further versatility of the "bio"surfactants. The resulting PS-NPs were in the size range between 80–100 nm with a narrow size distribution. The size of the PLLA and PP-NPs was between 100–200 nm with a broader size distribution than for PS-NPs. For all synthesized nanoparticles a good stability without any aggregation or flocculation was observed.

In summary, the classical surfactant SDS can be effectively substituted by the biocompatible and degradable amino-acid based surfactants SLS, SLG and SMS to produce a variety of nanoparticle dispersions for many bio-related applications.

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