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Cytotoxicity of PMMA-Based Nanoparticles Synthesized Adopting SDS and Tween 80

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Summary: in this work the cytotoxicity of PMMA-based nanoparticles against mouse mammary cancer cells (4T1) has been investigated. NPs have been synthesized using either monomer starved semi-batch emulsion polymerization (MSSEP) or standard batch emulsion polymerization (BEP) processes adopting potassium persulfate (KPS) as initiator and two different emulsifiers: sodium dodecyl sulfate (SDS) and Tween 80. The toxicity of NPs produced using SDS has been confirmed in *in vitro* experiments while it has been found that NPs stabilized with Tween 80 show a good biocompatibility. Moreover, the absence of toxicity of NPs in which the SDS is substituted with Tween 80 adopting ion exchange resins (IER) has been proved. Finally the biocompatibility of the sulfate chain end groups coming from the adopted initiator has been assessed.

Keywords: biocompatibility; nanoparticles; PMMA; SDS; surfactants

Introduction

Polymeric materials have been studied extensively in the last years for their biomedical applications ranging from resorbable sutures to scaffolds and from tissue engineering to vectors for drug delivery applications. Therapeutics nanoparticles (NPs) have many potential advantages such as the ability to target specific tissues or cells and the possibility to deliver drugs through biological barriers.[1] Among all the possible synthetic routes, the most common processes adopted to obtain polymeric NPs are the nanoprecipitation,[2] the solvent evaporation,[3] and the emulsion polymerization, which has the advantage of being an organic solvent-free process. Emulsion polymerization requires the use of a surfactant in order to stabilize the NPs that can cause serious toxicity issues.[4] In particular, sodium dodecyl sulfate (SDS) which is one of the most used surfactants, was demonstrated to be toxic to cells because of its capacity to bind to various bioactive macromolecules such as proteins, peptides and DNA or to insert into various cell fragments (i.e. phospholipid membranes) causing misfunction.[5,6] On the other hand, the use of SDS in both monomer starved semi-batch emulsion polymerization (MSSEP), in which the monomer is slowly fed in the reactor in order to ensure starved condition,[7] and batch emulsion polymerization (BEP) processes, allows obtaining well defined monodispersed NPs tunable in terms of dimension and ζ potential.[8] Recently, the possibility to synthesize tunable fluorescent PMMA-based NPs for imaging applications has been reported in literature.[9] In the latter publication Dossi et al. synthesized polymer NPs using KPS as initiator, SDS and Tween 80 as surfactants and adopting both MSSEP and BEP processes. In this work the toxicity of PMMA-based NPs has been investigated starting from the results reported by Dossi et al.. In particular the cytotoxicity of the NPs obtained adopting SDS and Tween 80 has been evaluated along with the effects of the SDS substitution with Tween 80 using ion exchange resins (IER) as reported in Scheme 1. The

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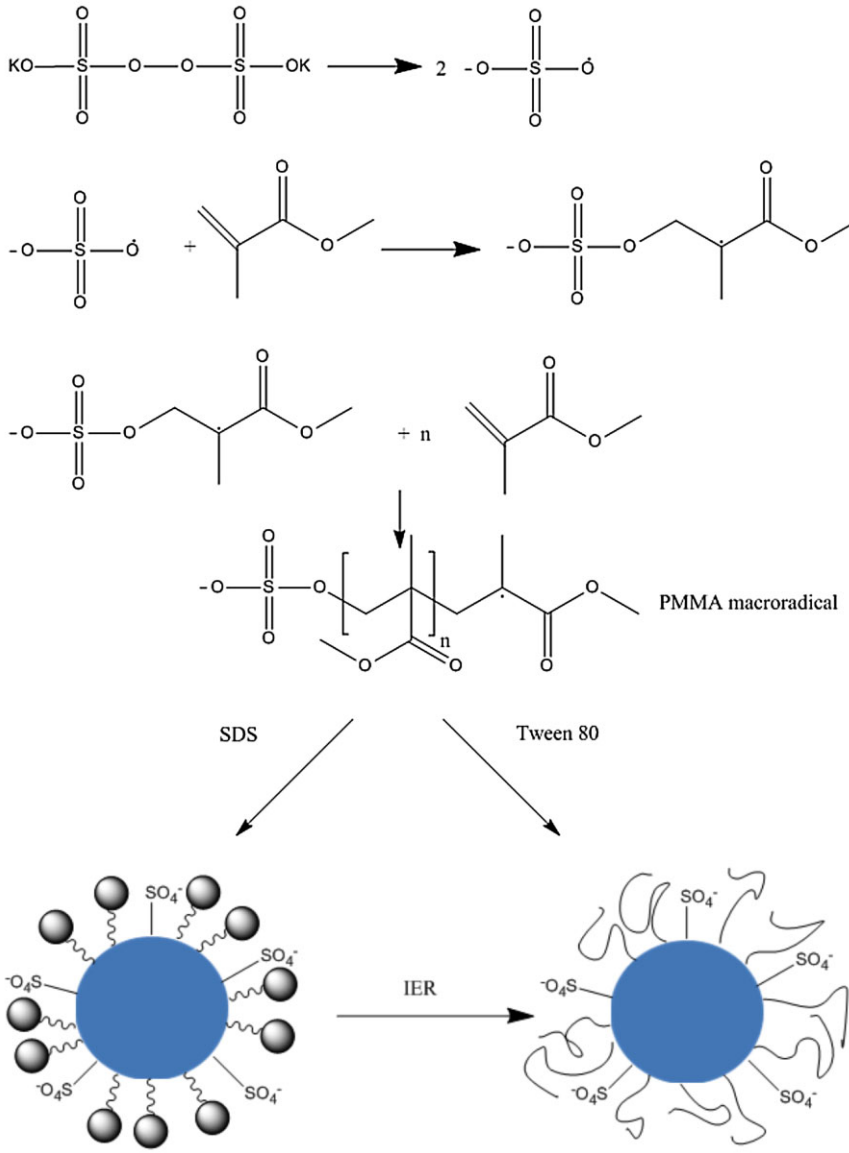

Scheme 1.
Production route of PMMA-based NPs.

cytotoxicity of the NPs has been evaluated in *in vitro* experiments on a mouse mammary tumor cell line 4T1.

Experimental Part

Materials

Methyl methacrylate (MMA) containing less than 30 ppm of hydroquinone-monomethyl-ether (MEHQ) as an inhibitor (99% purity, Sigma-Aldrich), sodium dodecyl sulfate (SDS) powder (assay 90%, Merck), polyoxyethylenesorbitanmonooleate (Tween80) (Sigma-Aldrich), potassium persulfate (KPS) (ACS reagent, >99% purity, Sigma-Aldrich), rhodamine B (RhB) (Sb sensitivity <0.1 ìg · mL-1, Carlo Erba reagents), dicyclohexylcarbodiimide (DCC) (99% purity, Sigma-Aldrich),

dichloromethane (DCM) containing 50–150 ppm of a stabilizer (>99.9% purity, Sigma-Aldrich), 2-Hydroxyethyl methacrylate (HEMA) containing up to 50 ppm of hydroquinone-monomethyl-ether (MEHQ) as an inhibitor (>99% purity, Sigma-Aldrich), ion exchange resins (Dowex Marathon MR-3, Supelco Analytical), cell medium (DMEM/F12, Biowest), fetal bovine serum (Lonza), L-glutamine (Biowest) and 0.05% trypsin-0.02% EDTA in PBS (Biowest) were all used as received.

Nanoparticles Synthesis and Characterization

The NPs synthesis and characterization is reported in detail in a recent publication.[9] Briefly, NPs synthesis has been carried out using a 100 ml three necks glass flask which was put in an oil bath at 70 °C under magnetic stirring. KPS was used as water soluble initiator and SDS or Tween 80 as emulsifiers. The monomer was either put in the flask at the beginning of the reaction (BEP) or fed with a selected injection rate using a syringe pump (NE 300, New Era Pump Systems). Before the injection of the initiator the system was kept under a nitrogen atmosphere in order to avoid the presence of oxygen. For all the syntheses 5 g of MMA were copolymerized with 0.04 g of a fluorescent monomer composed by a HEMA molecule covalently bound with RhB, in 100 ml of deionized water as reported by Dossi et al..[9] The mass ratio between initiator and water was kept constant and equal to 0.1%, while feeding rate in the MSSEP process was set to 6 ml/h. Two SDS-to-monomer ratios were selected, namely 2% for the MSSEP process and 0.5% for the BEP one; while the Tween 80-to monomer ratio has been set to 20% using the MSSEP process.

As already reported, a procedure that involves IER to substitute SDS with Tween 80 was applied. More in detail, 10 ml of colloidal suspension were mixed in a glass vial with an amount of Tween 80 equal to that of SDS; the suspension was then leached twice in a glass column (15 cm height, 1 cm diameter) containing 3 g of ion exchange resins.[10] The effectiveness of the surfactants exchange by adopting a mixture of cationic and anionic ion-exchange resins is reported in literature.[11,12] The average particle size (D_p), poly dispersity index (PDI), and ζ-potential were measured by dynamic laser light scattering (DLLS, Zetasizier Nano Series, Malvern Instruments).

Cells Culture, Cytotoxicity Study and Imaging

4T1 cells were grown in high glucose DMEM/F12 (Biowest) supplemented with 10% fetal bovine serum (Lonza) and 2% L-glutamine (Biowest) and maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were grown as monolayers in T25 tissue flasks (Iwaki) and routinely subcultured twice weekly, detaching them using 1 ml 0.05% trypsin-0.02% EDTA in PBS (Biowest). Trypsin activity was stopped using culture medium. All detached cells were counted using a Coulter counter ZM (Coulter Electronics). NPs toxicity on 4T1 cells was tested counting the cells with the Coulter counter at different times of incubation with NPs. 4T1 cells were seeded in 6-well plates (BD Falcon™) at a density of about 20 cells/mm^2. 24 h after seeding, cells were incubated with different NPs concentrations, equal to $1.15 \cdot 10^{12}$ NPs/ml for Tween 80 and $8 \cdot 10^{11}$ and $4 \cdot 10^{11}$ NPs/ml for SDS. After 24 h and 48 h of incubation, cells were washed with PBS, detached with trypsin and counted. Three replicates for each sample were considered. For imaging purpose 4T1 cells were seeded in a 6-well plate and 24 h later were incubated with NPs for 24 h (following the same experimental procedure used to test NP toxicity). At the end of incubation, images were acquired in phase contrast and red fluorescence using an inverted microscope IX81 (Olympus) with an UPlanFLN 10× (Ph1) objective with 0.30 NA.

Results and Discussions

Nanoparticles Synthesis and Characterization

Nanoparticles have been synthetized as reported elsewhere[9] and characterized with dynamic light scattering in terms of size, ζ- potential and PDI and the obtained results are reported in Table 1. Obtained results clearly show, as expected, that SDS is a much more effective surfactant than Tween 80 for the synthesis of NPs through emulsion polymerization. In fact, the use of an SDS amount ten times smaller than Tween 80 leads to smaller NPs. There is also a significant difference in the values of the NPs ζ-potential as a function of the surfactant adopted. In particular NPs synthesized with SDS present a ζ-potential of about −50 mV while the use of Tween 80 leads to values of about −20 mV. The residual charge over these latter NPs is due to the presence of sulfate groups coming from the KPS that initiates the free radical polymerization. Since there is an ongoing discussion in literature about cellular toxicity of SDS the NPs synthesized adopting this surfactant were processed in order to exchange SDS with Tween 80.[9] The characteristic of the NPs synthesized with SDS (Sample A1 and A2) and then stabilized with Tween 80, after the anionic surfactant removal, are reported in Table 2.

Results reported in Table 2 show a concrete reduction of the absolute value of the ζ- potential that qualitatively confirms the effective SDS removal from the NPs surface. It is also worth noticing that the dimension of the NPs does not change significantly during the process proving the absence of an aggregation process during the surfactant substitution. Nevertheless, a slight increase in the polydispersity index is observed and this evidence is probably due to the fact that the NPs hydrodynamic radius is slightly increased non-homogeneously because of the presence of the steric surfactant (Tween 80).

Table 2.
Characteristics of the NPs after surfactant substitution.

Sample	Feed	D_p [nm]	PDI	ζ [mV]
B1	MSSEP	55.4	0.12	−33
B2	BEP	95.5	0.04	−22

Cytotoxicity Results

The cytotoxicity of NPs synthesized with SDS and Tween 80 was investigated on 4T1 cells. As a first result, it was found that NPs obtained adopting SDS are extremely toxic for the selected cells. In fact, after an incubation of 24 h at the lowest NPs concentration of both Sample 1 and 2 no cells survival was detected. On the other hand, NPs produced with Tween80 (Sample A3) showed a good biocompatibility as reported in Figure 1. Moreover, in order confirm the reproducibility of the obtained results, another batch of NPs (Sample $A3_{bis}$) synthesized adopting Tween 80 under the same conditions of Sample 3 was tested. Results reported in Figure 1 show that, after 24 hours, no significant changes in the number of the cells can be found for both Sample A3 and $A3_{bis}$. After 48 hours there is a very slight decrease in cell proliferation in particular for the Sample $A3_{bis}$, even if this evidence cannot be related to NPs toxicity.

In Figure 2, images of the 4T1 cells in contact with the NPs (Sample A3 and B1, upper and lower panels, respectively) are reported. In the left panels, phase contrast images allow to appreciate the cell morphology, while in the right panels the

Table 1.
Size, PDI, and surface ζ potential for the produced NPs.

Sample	Surfactant	Surf./Mon. (% w/w)	Feed	D_p [nm]	PDI	ζ [mV]
A1	SDS	2	MSSEP	50.2	0.08	−52.5
A2	SDS	0.5	BEP	89.8	0.03	−42.3
A3	Tween 80	20	MSSEP	92.5	0.02	−18
$A3_{bis}$	Tween 80	20	MSSEP	85.2	0.03	−15.4

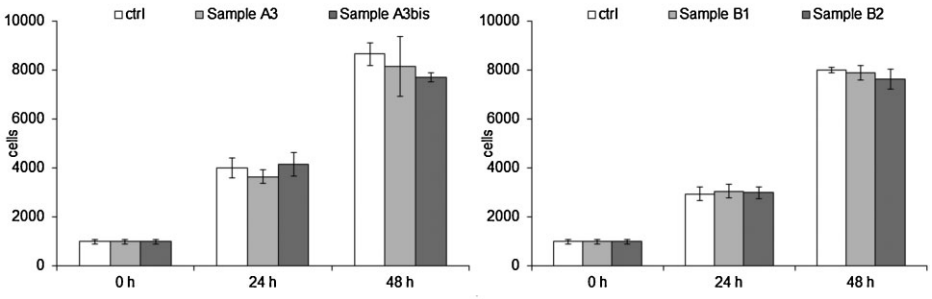

Figure 1.
Cell proliferation of controls (ctrl) and cells exposed to different NPs concentrations. Left panel: NPs synthesized adopting Tween 80 (Sample A3 and $A3_{bis}$), right panel: NPs after the SDS substitution (Sample B1 and B2). The cell number was normalized to 0h and set equal to 1000.

fluorescence of RhB covalently bound to the polymer permits the detection of internalized NPs. In particular, for both kinds of NPs, the fluorescence signal was localized in the perinuclear region of the cells, demonstrating that NPs are able to enter the cells but cannot penetrate into the nuclei.

Once the biocompatibility of PMMA-based NPs synthesized with Tween 80 was

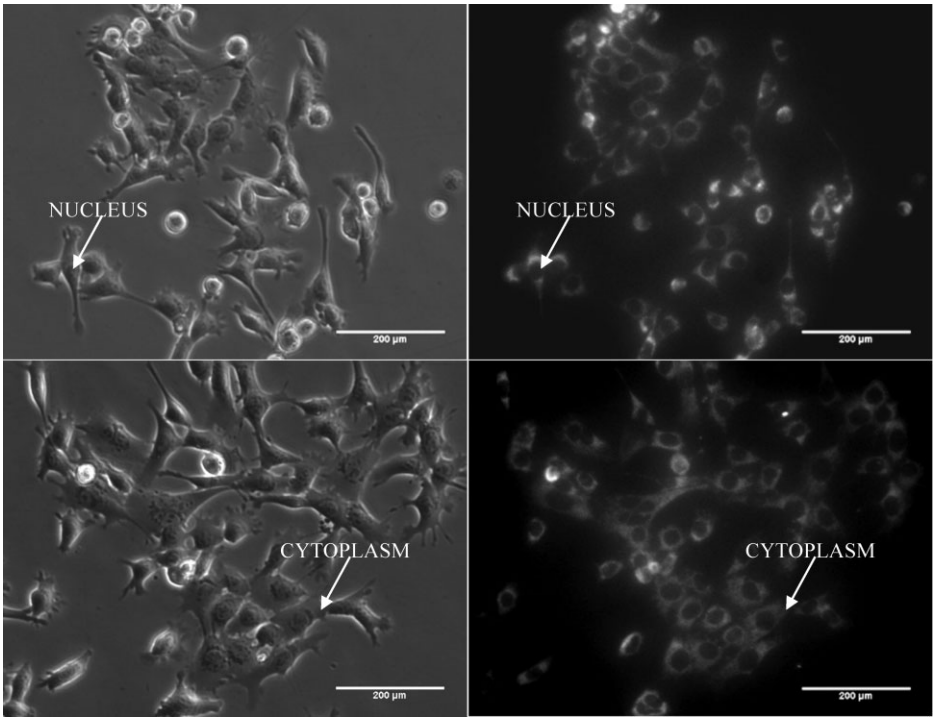

Figure 2.
Images of the cells in contact with the NPs (Sample A3, upper panels and Sample B1, lower panels): left panels, phase contrast images; right panels, image in red fluorescence (λ_{exc}: 572/23 nm, λ_{em}: 628/28 nm) of the same field of view. Cell autofluorescence in the red channel is neligible, thus the fluorescence signal detected in the cytoplasmic region is due to the presence of internalized NPs.

confirmed, the effects on cells proliferation of the NPs obtained after the SDS substitution were investigated. The relative cytotoxicity results performed on Sample B1 and B2 are reported in Figure 1 (right panel). Obtained results clearly prove the complete biocompatibility of the NPs synthesized with SDS and stabilized with Tween 80 after the anionic surfactant removal. Moreover, since no biological differences between the control (ctrl) and the two samples analyzed were found, it is possible to conclude that the size of NPs does not affect their growth. As an indirect evidence, it is also possible to conclude that the procedure adopted to remove the SDS is effective since no toxicity for both Sample B1 and B2 was found. As a final consideration it is worth noticing that the NPs obtained after the SDS substitution are characterized by a ζ-potential intermediate between the values obtained when SDS and Tween 80 are used (-33 and -22 mV, respectively). The absence of residual SDS leads to conclude that the higher ζ-potential of Sample B1 and B2 is due to a lower surface charge screening operated by the Tween 80 compared to the one measured for Sample A3 and $A3_{bis}$. As a result, the absence of toxicity found for Sample B1 and B2 along with the considerations reported above, proves that the biological effect of the sulfate groups coming from the initiator is negligible.

Conclusion

In this work the cytotoxicity of PMMA-based nanoparticles synthesized adopting SDS and Tween 80 was investigated. Obtained results on 4T1 murine cell line had shown a clear toxicity related to SDS while a complete biocompatibility for NPs stabilized with Tween 80 was found. Moreover experiments performed after the SDS removal and its substitution with Tween 80 indicated that no cytotoxic effects can be ascribed to the sulfate groups coming from the initiator.

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