IJPPR
INTERNATIONAL JOURNAL OF PHARMACY & PHARMACEUTICAL RESEARCH
An official Publication of Human Journals
ISSN 2349-7203
HUMAN

Human Journals
Research Article
June 2018 Vol.:12, Issue:3

In-Vitro Cytotoxicity of Nanoencapsulated Sunscreen Agents Obtained by Pickering Emulsion Polymerization

IJPPR
INTERNATIONAL JOURNAL OF PHARMACY & PHARMACEUTICAL RESEARCH
An official Publication of Human Journals

P. Leo[a]; N. N. P. Cerize*[a]; L. O. Feitosa[a], R. Gavioli[a], R. de S. Gois[a], B. N. M. Miranda[a], A. M. Oliveira[a]

[a]Institute for Technological Research, Group for BioNano Manufacturing, Av. Prof. Almeida Prado, 532, 05508-901, São Paulo, Brazil.

Submission: 20 May 2018
Accepted: 27 May 2018
Published: 30 June 2018

HUMAN JOURNALS
www.ijppr.humanjournals.com

Keywords: Polymeric nanoparticles Drug delivery systems Cytotoxicity Phototoxicity Sunscreen Pickering emulsion polymerization

ABSTRACT

Nanoparticulate materials have increased its applications in commercial products, mainly cosmetic. Polymeric nanoparticle to be used as carrier substance must have suitable chemical and physical properties and does not induce toxic reactions. Poly(methyl methacrylate) (PMMA) and polystyrene (PSty) are polymers with a potential to be used in the preparation of nanoparticles and used as a carrier system for drug delivery. This requires a better understanding of the interaction of these polymers with different organisms. In this study, the cytotoxicity and phototoxicity profile of the PMMA and PSty nanoparticles loaded with sunscreen agents, as 3-benzophenone (BZ-3) and octyl methoxycinnamate (OMC), on murine fibroblasts cells were measured by standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and neutral red uptake assay. PMMA nanoparticles were also evaluated by morphological transformation assay. The results showed that the PMMA and PSty nanoparticles are nontoxic and could be useful for various *in-vivo* and *in-vitro* biomedical applications.

1. INTRODUCTION

Lately, nanomaterials have been increasingly incorporated into consumer products, taking advantage of its special properties regarding its size, permeation capability and efficiency and somewhat beneficial marketing, especially regarding cosmetics. [1] Correspondingly, the field of nanotoxicity has grown significantly in order to address concerns (both public and regulatory) regarding the potential effects to the environment and human health of nanoparticle technology use. [1,2]

This research should maintain the future, and intensify debates, since the information concerning the potential hazards related to nanoparticle exposure is still rare. In particular, biological applications that employ nanoparticles to drug delivery have attracted much attention [3]. Numerous studies have investigated the harmful implications that nanomaterials can bring to human health and the environment [4]. These studies indicate that the nanoparticle-organism interaction is influenced by the physical and chemical properties of the nanomaterial, such as size, composition, shape, surface charge, potential of agglomeration, and solubility. Some of these attributes can modify the interaction of the nanoparticles with the organism and environment. Even a biocompatible and biodegradable material in its normal dimensions could have different reactions when in the nanoscale [5], for instance, achieving natural protective barriers (skin, mucosa) and reaching cells and organelles what is impossible in macro scale. Therefore, at the moment it is impossible to guarantee the security of nanoparticulate materials regarding their macro composition [6-8], what encourage safety and nanotoxicity studies.

A limited number of *in-vitro* studies have also been performed to assess the toxicities of the nanoparticles using different cellular systems and test methods [9, 10]. However, published toxicity data are still considered inadequate to earn a full understanding of the potential toxicity of these nanoparticles. Further studies are needed to clarify the risk of these materials as well as their application for human use [11-13].

Recently *in-vitro* methods have shown a significant potential for assessing the toxicity of environmental and occupational health risks [14-17]. One of the important methods is the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay, which is based on the ability of viable cells to convert a soluble tetrazolium salt to a formazan product, catalyzed by mitochondrial dehydrogenase enzymes and is, therefore, a measure of cell viability. Another is the NRU (neutral red uptake) assay, in which viable cells incorporate

the dye, and bind the supravital dye neutral red in the lysosomes. These methods have proven to be user-friendly, rapid and highly sensitive [18-21]. Another important evaluation regarding nanoparticles' use is their carcinogenic potential that can be investigated using the cell transformation assay.

The aim of this study was to evaluate cytotoxicity, phototoxicity, and carcinogenicity of nanoparticles composed by the polymers poly (methyl methacrylate) (PMMA) and polystyrene (PSty) synthesized via emulsion polymerization using *in vitro* assays. [22] Biological parameters that imply on safety insurance for in vivo application such as particle size and polydispersity index, zeta potential, morphology and total solid content were investigated. Cytotoxicity tests were assessed in mouse fibroblasts (NCTC-929) and Balb/c-3T3 using two viability assays, MTT, NRU, and phototoxicity protocols [23,24]. Carcinogenicity potential was evaluated by morphological cell transformation assay (CTA) under an optical microscope (OM) which allows identifying a carcinogenic substance by inducing morphological cell change [25,26].

2. MATERIALS

The analytical grade monomers used were Methyl Methacrylate (MMA) and Styrene (Sty) which were gently supplied by BASF Brazil S/A. The Styrenesulfonic acid sodium salt hydrate (StySO3) (Aldrich) Hydroethylmethacrylate (HEMA) (Aldrich, 99%) were used without previous purification. As sunscreen agents were used 3-benzophenone (BZ-3) and octyl methoxycinnamate (OMC). The Sodium Persulphate (Anidrol, 98%) was used as an initiator and colloidal silica pre-dispersed in water (Nalco, 31.25% w/w) was used as a protective colloid. The other reagents were used as received.

3. METHODS

Nanoparticles preparation by emulsion polymerization

The polymerization reactions were conducted in a water jacketed glass reactor (150 mL vessel) equipped with a reflux condenser and thermostatic bath (Lauda, model E100). Initially, the reactor was loaded with the colloidal silica dispersion and heated up to 70 °C. After that, the monomer was added and the initiator dissolved in a water quantity. The monomer to initiator mass ratio was set equal to 100 for all tested reactions and deionized water was added to complete 110 g (total mass). The reactions were carried for four hours and the obtained

products were cooled and characterized just after the completion of the described protocol. Table 1 shows the formulations and experimental conditions used in the present work.

Nanoparticles Characterization

Particle size and polydispersity index

The mean particle sizes were determined by the means of the dynamic light scattering technique using a DelsaNano C model, Beckmann Coulter equipment. All measurements were performed in triplicate. Any dilution required to adjust the obscuration range compatible with the technique was performed by adding distilled water.

Zeta potential

The Laser Doppler Electrophoresis technique was used to determine the velocity of charged particles under the influence of an applied electric field (electrophoretic mobility). All measurements were performed in a single point mode and repeated fifteen times to ensure a representative value expressed by its mean and a standard deviation. Zeta potential (ZP) was calculated from the measured electrophoretic mobility considering the Smoluchowsky theory. The equipment used was Zetasizer-Nano series, Malvern. Distilled water was used as the dispersing medium and each sample was appropriately diluted to ensure a significant signal/noise ratio detected by the photon correlator.

Total Solids Content

The degree of monomer conversion after polymerization for each of the reactions (see Table 1) was estimated from the experimentally determined total solids content (TSC). The measurements were performed by the means of gravimetric analysis using an oven with air circulation (Nova Ética model 400/3ND) and a halogen light thermogravimetric balance (Mettler Toledo model HB43-S). The samples were weighed before and after oven drying for 1h at 105 ºC. The initial weight of each sample was approximately 1.0 g once considered the tare weight of the aluminum crucibles and the samples were dried to constant weight under halogen light.

High-Resolution Scanning Electron Microscopy

The morphological aspects of the nanoparticles were further characterized using the high-resolution scanning electron microscopy technique (HR-SEM). A field emission microscope model Quanta 3D (FEI Instruments) was used and the system operated at high vacuum mode and an accelerating voltage of 20 kV. The samples were sputter coated with gold-palladium alloy any charge-up effect.

Table 1: Formulation and experimental conditions tested in the different polymerization reactions.

Sample ID	MMA (g)	Sty (g)	BZ-3 (g)	OMC (g)	NaSty (g)	HEMA (g)	Silica (g)	Water (g)
NTX 31	----	5.02	0.50	0.50	0.16	0.17	3.32	45.92
NTX 32	5.15	----	0.50	0.51	0.16	0.17	3.33	46.07

Cell lines and culture conditions

Mouse fibroblasts NCTC-929 and Balb/c 3T3 cells were purchased from Adolfo Lutz Institute - SP and BCRJ (Bank Cell Rio de Janeiro) respectively.

These cell lines were grown and maintained using suitable media (DMEM, SigmaUSA) supplemented with 10% fetal bovine serum (FBS, Invitrogen). Cells were seeded at 200,000 cells/T-25 flask in a total volume of 6 mL. When confluent, all the cells were detached using trypsin-EDTA (Sigma – USA)

Evaluation of Cytotoxicity Using the MTT and NRU Assay

Citotoxicity evaluation was assessed by monitoring the conversion of MTT to formazan and neutral red uptake assay. In brief, mouse fibroblasts cells NCTC-929 and Balb/c 3T3 (100 µL; 1×105 cells.mL^{-1}) were seeded into 96 well microtitre plates as before and left to adhere for 24 h. The next day, the medium was removed from the wells and replaced with sterilized complete medium 5% FCS containing nanoparticles serially diluted at concentrations ranging from (15.0 to 0.078) mg.mL^{-1} (100 µL/well).

The plates were then incubated with PMMA and PSty nanoparticles for 24 h. Afterwards, the medium was replaced with complete media 5 % FCS cointaining MTT (1 mg.mL^{-1}). Plates

were incubated for a further 2 h. Then the medium was removed and isopropanol (100 µL) added before an incubation of 30 min at 37 ºC. Finally, the absorbance at 570 nm of the plates was read with the Multiskan plate reader spectrophotometer (Titertek, USA). Media absorbance of cells exposed to medium only was taken as 100% cell viability (the negative control). Inhibition of growth of cells was calculated from the relative absorbance of untreated control cells at 570 nm and expressed IC 50.

The cells were also incubated for 3 h with a medium containing neutral red dye (50 µg.mL^{-1}). The cells are subsequently washed, the dye was extracted from each well using ethanol/ acetic acid/water (50%/1%/49%) and the absorbance was read using a 540 nm spectrophotometer.

The 3T3 Neutral Red Uptake (NRU) Phototoxicity

Following literature protocols [24], duplicate 96-well monolayers of 3T3 fibroblasts were exposed to serial dilutions of a test material. One of the plates was exposed to 5 J/cm^2 UVA(Q-SUN XE-1 XENON TEST CHAMBER) while the other plate was kept in the dark for 50 minutes. To assess viability, the neutral red uptake (NRU) by cells exposed to the test chemical in the presence of UVA exposure was compared to the NRU by cells exposed to the test chemical in the absence of UVA exposure.

Morphological cell transformation assay

According the document [25], a total of 1.5 x 103 cells.mL^{-1} in 10 mL of complete culture medium were seeded in 100 Petri dish (Corning,USA). After 24 h, the medium was changed to those containing selected concentrations of NTX-31 or NTX-32 (5 replicates each concentration). In particular, we used (2.0, 5.0, 13.0 and 20.0) mg to NTX-31 nanoparticles and (0.5, 3.0, 8.0 and 10.0) mg.mL^{-1} to NTX-32 corresponding to IC20, IC30, IC50 and IC80. Methylcholanthrene was used as a positive control (1, 2, 3 and 4) µg.mL^{-1}.

After treatment during 72 h, the media was changed and cultures were maintained for 5 weeks, with the media renewed twice a week with 2 % FCS media. In the end, the cells were fixed with methanol for 10 min and stained with Giemsa (10%) for 30 min. Type III foci were manually scored for morphological transformation under stereomicroscope, as described by Sasaki et al (2012).[26]

Experimental data were analyzed by Fisher's exact test considering the number of type III foci in the treatments and the surviving cells compared to the corresponding negative control (untreated cells). Only $p > 0.05$ was considered statistically significant.

Statistical analysis

When at least 2 viability values were below 50% of control condition, the IC50 (toxic concentration 50, concentration of particles inducing 50% cell mortality) was calculated using 3T3 Phototox software (logarithmic transformation of X-values and nonlinear regression - sigmoidal dose-response analysis with variable slope- with bottom and top constraints set at 0 and 100 respectively).

4. RESULTS

Manufacturing process

Here, two different monomers were evaluated in the Pickering emulsion polymerization process, Sty (NTX 31) and MMA (NTX 32). Both monomers producednanoparticles with spherical geometry (Figure 1) with average particle size 91.1 and 112.27, respectively, and low polydispersity, as shown in Table 2. Zeta potential was found to be negative, with values of -32.60 for NTX 31 and -33.20 for NTX 32.

The solids content was 13.77% for NTX 31 and marginally higher for NTX 32. Comparing this data to the expected TSC for each sample, and assuming that non-reacted monomer comprises the majority of the remaining volatile material in the samples, it was possible to estimate the polymerization efficiency for both materials. This polymerization efficiency was estimated to be higher for NTX 32, this sample was expected to contain less residual MMA monomer.

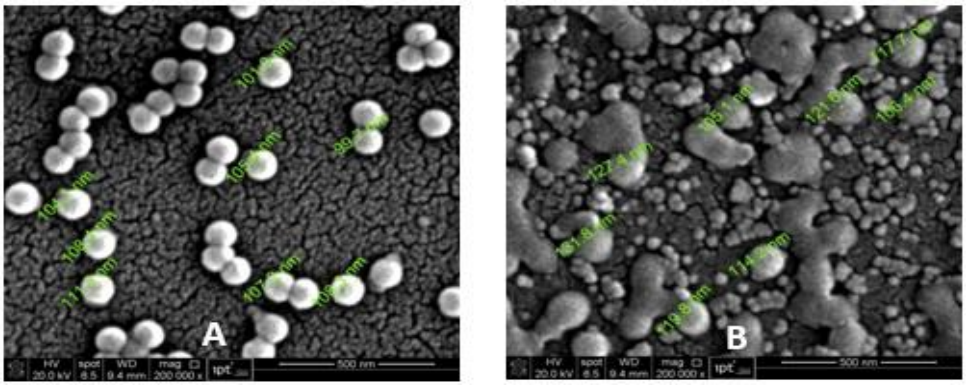

Figure 1: Scanning electron microscopy of (A) NTX 31 and (B) NTX 32 nanoparticles, respectively.

Table 2: Polystyrene and PMMA nanoparticles characterization.

Sample ID	Dm	IP	TSC*(%)	PE**	ZetaPotential
NTX 31	91.1	0.019	13.77	84.31	- 32.60
NTX 32	112.7	0.006	14.15	95.26	- 33.20

*TSC: total solid content; PE: polymerization efficiency.

Cytotoxicity studies

Obtained results indicated non-cytotoxicity potential for the PSty NXT 31 and PMMA NXT 32 nanoparticles on two murine cell lines. The cytotoxicity data of the two nanoparticles with the MTT and NR assay method are presented in Figures 2, 3, 4 and 5.

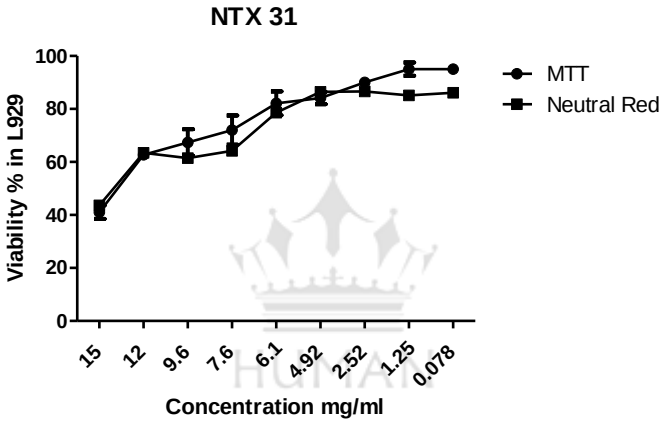

Figure 2: Concentration-cell viability curves of NTX 31 Styrene-NPs following 24-hour exposure on murine fibroblasts NCTC-929 using the MTT and NRU assay.

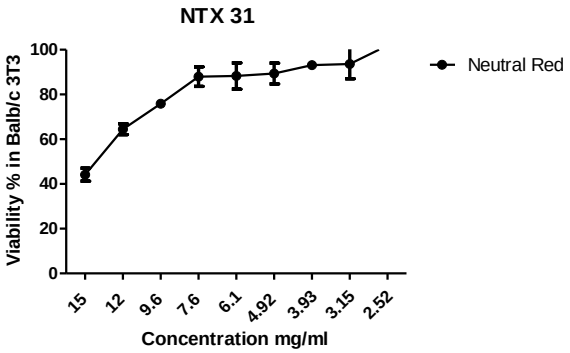

Figure 3: Concentration-cell viability curves of NTX-31 Styrene-NPs following 24-hour exposure on murine fibroblasts Balb/c – 3T3 using the NRU assay.

Table 3: NTX 31 and NTX 32 IC50 value obtained using two cell line and two vital dye.

Sample ID	IC50 VN 3T3	IC50 VN 929	IC50 MTT 3T3
NTX 31	11.96 ± 0.5 mg/mL	12.6 ± 0.32 mg/mL	11.44± 1.04 mg/mL
NTX 32	6.5 ± 0.6 mg/mL	6.15 ± 0.5 mg/mL	7.8± 0.65 mg/mL

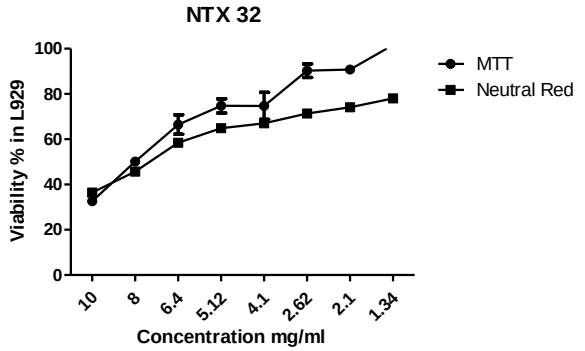

Figure 4: Concentration-cell viability curves of NTX-32 PMMA-NPs following 24-hour exposure on murine fibroblasts NCTC-929 using the using the MTT and NRU assay.

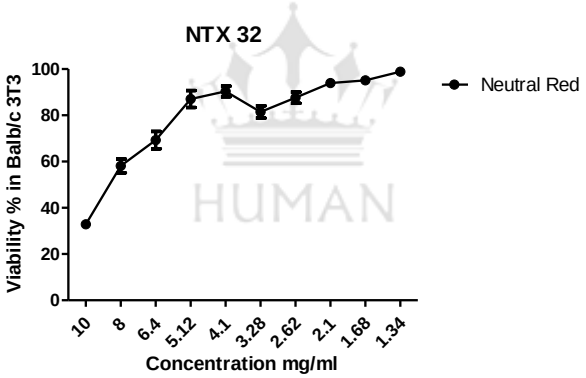

Figure 5: Concentration-cell viability curves of NTX-32 PMMA-NPs following 24 hours exposure on murine fibroblasts Balb/c – 3T3 using the NRU assay.

For both nanoparticle samples evaluated, IC50 values calculated (respectively concentration corresponding to 50% viability) were similar on the cell types and independent of assay method as shown in Table 3.

The IC50 values of NTX 31, were found to be between 11.4 – 12.6 $mg.mL^{-1}$. NTX 32 nanoparticle showed IC50 between 6.1 – 7.8 $mg.mL^{-1}$ (Table 3). According to [27] substances with IC50 above of 0.175 $mg.mL^{-1}$ were considered as nonirritant.

Phototoxicity 3T3 NRU

No toxicity induced by visible irradiation was observed when Balb/c 3T3 cells were exposed to 5 J/cm2 UVA. The Photo-Irritation-Factor (PIF) and Mean Photo Effect (MPE) obtained are shown in Table 4. Based on the validation study, a test substance with a PIF < 2 or an MPE < 0.1 predicts no phototoxicity [28]. These results support the possibility of using these nanoparticles in products such as cosmetics.

Table 4: NTX 31 and NTX 32 phototoxicity values.

Sample ID	PIF	Toxicological Probability	MPE	Toxicological Probability
NTX 31	0.917	Non toxic	0.015	Non toxic
NTX 32	1.780	Non toxic	0.022	Non Toxic

Cell transformation

In this assay concentrations above and below the IC 50 were calculated by 3T3-phototox software, using MTT or NRU results. The concentration used were 2.0, 5.0, 13.0 and 20.0 mg for PSty-NTX 31 nanoparticles and 0.5, 3.0, 8.0 and 10 mg.mL-1 for PMMA-NTX 32. For the concentrations tested, none of the particles induced significant cellular changes.

5. DISCUSSION

Polymeric nanoparticle must have suitable mechanical properties, appropriate degradation time and does not induce toxic reactions to be used as carrier systems. The polymers used as biomaterials must also be biocompatible [29,30] and functionally active. The poly(methyl methacrylate)e (PMMA) is a polymer often used in the preparation of nanoparticles and used as a carrier system for substances widely applied to biomedical devices [31]. The PMMA systems consist of nanoparticles with good biocompatibility, which in its bulk form is more toxic than when nanoparticles [32]. In [33] was showed that the polymeric film of PS, PMMA, 1:1 PS/PMMA was suitable for promoting cell adhesion and proliferation, without the need for any surface modification but they suggested that in vivo cytotoxicity and cell adhesion tests are necessary to establish a more precise and direct evaluation of the advantages and disadvantages of PS/PMMA systems [31]. The toxicity of functionalized PSty nanoparticles have been evaluated toward the yeast *Saccharomyces cerevisiae* and nanoparticles with negative surface charge showed littler to no toxicity [34].

In this work, both PSty NTX 31 and PMMA NTX 32 nanoparticles showed no adverse effects on the fibroblast murine cells Balb/c-3T3 and NCTC929, revealing to be biocompatible. The concentrations to obtain the IC50 values are quite high for a substance to be considered critical for use in cosmetics. PMMA is widely used as coating nanoparticles for providing biocompatibility.

The cytotoxicity and phototoxicity profile of the nanoparticles on murine fibroblasts as measured by standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and neutral red uptake showed that the particles are nontoxic or phototoxic neither carcinogenic and may be useful for various *in-vivo* and *in-vitro* biomedical applications.

Nanomaterial usage will continue to increase rapidly and widely in areas such as cosmetics, pharmaceuticals, and other industrial applications. Accurately assessing the toxicity and safety of these nanomaterials to human health is of upmost importance. The present study focused too on investigating the potential risk of nanomaterials at the microscopic cellular level by morphological cell transformation assay. This assay can help revealing general mechanisms of toxicity and characterizing exposure to nanomaterials.

Finally, for the industry, the ultimate aim of nanobiotechnology is to translate the generated knowledge into an economically viable and sustainable application. The perspectives are bright with a multitude of potential applications including drug delivery, new materials, engineering tissue [35,36]. However, most research and development activities are still focused on gathering understanding, concept development, providing proof of concept and making first prototypes. In order to address the nanotoxicity and the understanding of the mechanisms that are involved in cytotoxicity of nanoparticles remains a big challenge. This work aims to contribute to the art state of adapting methodologies to evaluate polymeric nanoparticles toxicity to biomedicine including theranostics system [37].

6. CONCLUSION

In summary, cell exposure of poly(methyl methacrylate) and polystyrene nanoparticles produced concentration-dependent cell death for concentrations above the IC50, and it did not induce cell transformation. Therefore, PMMA and PSty nanoparticles can be considered nontoxic substances. Further studies are needed to investigate PMMA and PSty nanoparticles cytotoxicity to guarantee safety to drug delivery applications.

These results allow us to anticipate future applications in biomedical research such as drug delivery, diagnosis and therapeutical proposals, such as theranostics systems.

7. ACKNOWLEDGMENT

The authors gratefully acknowledge FIPT and IPT for infrastructure and financial support for this work.

8. REFERENCES

1 Vance ME, Kuiken T, Vejerano EP, McGinnis SP, Hochella MF, Rejeski D, et al. Nanotechnology in the real world: Redeveloping the nanomaterial consumer products inventory. Beilstein J Nanotechnol. 2015;6:1769-1780.
2 Maynard AD, Warheit DB, Philbert MA. The new toxicology of sophisticated materials: nanotoxicology and beyond. Toxicol Sci. 2011;120(1):109-129.
3 Parveen S, Misra R, Sahoo SK. Nanoparticles: a boon to drug delivery, therapeutics, diagnostics and imaging. Nanomedicine. 2012;8(2): 147-166.
4 Elsaesser A, Howard CV. Toxicology of nanoparticles. Adv. Drug Deliv. Rev. 2012; 64: 129-137.
5 Oberdörster, G. Toxicology of ultrafine particles: *in-vivo* studies. Philos Trans A Math Phys Eng Sci. 2000;358 :2719-2740.
6 Roiter Y, Ornatska M, Rammohan AR, Balakrishnan J, Heine DR, Minko S. Interaction of nanoparticles with lipid membrane. Nano Lett. 2008;8(3):941-944.
7 Zhang Y, Yang M, Portney NG, Cui D, Budak G, Ozbay E, et al. Zeta potential: a surface electrical characteristic to probe the interaction of nanoparticles with normal and cancer human breast epithelial cells. Biomed Microdevices. 2008;10: 321-328.
8 Shang L, Nienhaus K, Nienhaus GU. Engineered nanoparticles interacting with cells: size matters. J Nanobiotechnology. 2014;12: 1-11.
9 Sayes CM, Wahi R, Kurian PA, Liu Y, West JL, Ausman KD, et al. Correlating nanoscale titania structure with toxicity: a cytotoxicity and inflammatory response study with human dermal fibroblasts and human lung epithelial cells. Toxicol Sci. 2006;92: 174-185.
10 De Lima R, Feitosa L, Pereira AE, De Moura MR, Aouada FA, Mattoso LHC, Fraceto LF. Evaluation of the genotoxicity of chitosan nanoparticles for use in food packaging films. J Food Sci. 2010;75(6): N89-N96.
11 Hu, F.X.; Neoh, K.G.; Kang, E.T. Synthesis and in vitro anti-cancer evaluation of tamoxifen-loaded magnetite/PLLA composite nanoparticles. Biomaterials. 2006;27(33): 5725-5733.
12 Smuldersa S, Luytsa K, Brabantsb G, Golanskic L, Martensb J, Vanoirbeeka J, et al. Toxicity of nanoparticles embedded in paints compared to pristine nanoparticles, in vitro study. Toxicol Lett. 2015;232 (2): 333-339.
13 Tomankova K, Horakova J, Harvanovab M, Malina L, Soukupovad J, Hradilovad S, et al. Cytotoxicity, cell uptake and microscopic analysis of titanium dioxide and silver nanoparticles *in-vitro*. Food Chem Toxicol. 2015;82: 106-115.
14 Bernabeu E, Gonzalez L, Legaspi MJ, Moretton MA, Chiappetta DA. Paclitaxel-Loaded TPGS-b-PCL nanoparticles: *in-vitro* cytotoxicity and cellular uptake in MCF-7 and MDA-MB-231 cells versus mPEG-b-PCL nanoparticles and abraxane. J Nanosci Nanotechnol. 2016;16(1):160-170.
15 Rajiv S, Jerobin J, Saranya V, Nainawat M, Sharma A, Makwana P, et al. Comparative cytotoxicity and genotoxicity of cobalt (II, III) oxide, iron (III) oxide, silicon dioxide, and aluminum oxide nanoparticles on human lymphocytes *in-vitro*. Hum Exp Toxicol. 2016;35(2):170-183.
16 Chattopadhyaya S, Dasha SK, Tripathya S, Dasa B, Mandala D, Pramanikb P, et al. Toxicity of cobalt oxide nanoparticles to normal cells; an *in-vitro* and *in-vivo* study. Chemico-Biological Interactions. 2015;226: 58-71.
17 Promega Corporation. Cell Titer 96® AQueous non-radioactive cell proliferation assay, Technical Bulletin #TB169. Promega Corporation, 2005.

18 Kroll A, Pillukat M H, Hahn D, Schnekenburger J. Interference of engineered nanoparticles with *in-vitro* toxicity assays. Arch Toxicol. 2012;86(7):1123-1136.
19 Ivask A, Titma T, Visnapuu M, Vija H, Kakinen A, Sihtmae M, et al. Toxicity of 11 metal oxide nanoparticles to three mammalian cell types *in-vitro*. Curr Top Med Chem. 2015;15(18):1914-1929.
20 Costa C, Brandão F, Bessa MJ, Costa S, Valdiglesias V, Kiliç G, et al. *In-vitro* cytotoxicity of superparamagnetic iron oxide nanoparticles on neuronal and glial cells. Evaluation of nanoparticle interference with viability tests. J. Appl. Toxicol. 2016;36 (3): 361-372.
21 Mukherjeea SG, O'Claonadha N, Caseya A, Chambersa G. Comparative in vitro cytotoxicity study of silver nanoparticle on two mammalian cell lines. Toxicol In Vitro. 2012;26(2): 238-251.
22 Oliveira AM, Guimarães KL, Cerize, NNP. The role of functional monomers on producing nanostructured lattices obtained by surfactant-free emulsion polymerization – A novel approach. Eur Polym J. 2015;71: 268-278.
23 International Organization for Standardiation (ISO). ISO 10993-5:2009 Biological evaluation of medical devices - Part 5: Tests for cytotoxicity: *in-vitro* methods. 2009.
24 Organization for Economic Co-operation and Development (OECD). Test No. 432: In vitro 3T3 NRU phototoxicity test, OECD Guidelines for the Testing of Chemicals, Section 4, No. 432. OECD Publishing, 2004.
25 European Union Reference Laboratory for alternatives to animal testing (EURL-ECVAM). Balb/c 3T3 cell transformation assay – Prevalidation study report v. 2010.
26 Sasaki K, Bohnenberger S, Hayashi K, Kunkelmannb T, Muramatsu D, Phrakonkham P, et al. Recommended protocol for the BALB/c 3T3 cell transformation assay. Mutat Res. 2012;744: 30-35.
27 Bracher M, Faller C, Spengler J, et al. Comparison of in vitro cell toxicity with *in-vivo* eye irritation. Mol Toxicol.1988;4:561-570.
28 Spielmann H, Balls M, Dupuis J, et al. The international EU/COLIPA In vitro phototoxicity validation study: results of phase II (blind trial), part 1: the 3T3 NRU phototoxicity test. Toxicol In Vitro. 1988;12: 305-327.
29 Musyanovych A, Wienke JS, Mailander V, Walther P, Landfester K.. Preparation of biodegradable polymer nanoparticles by miniemulsion technique and their cell interactions. Macromol. Biosci. 2008;8;127-139.
30 Orefice, RL, Pereira, MM, Mansur HS. Biomateriais: Fundamentos e. Aplicações. Rio de Janeiro: Guanabara Koogan: 2006.
31 Bettencourt A, Almeida AJ. Poly(methyl methacrylate) particulate carriers in drug delivery J Microencapsul. 2012;29(4):353-367.
32 Mendes A, Hubber I, Siqueira M, Barbosa GM, Moreira DL, Holandino C, et al. Preparation and cytotoxicity of poly(methyl methacrylate) nanoparticles for drug encapsulation. Macromol Symp. 2012;319(1):34-40.
33 Melo A, Bet AC, Assreuy J, Debacheraand NA, et al. Adhesion of L929 mouse fibroblast cells on poly(styrene)/poly(methyl methacrylate) films. J. Braz. Chem. Soc. 2009;20: 1753-1757.
34 Miyazaki J, Kuriyama Y, Miyamoto A, Tokumoto H, Konishi Y, Nomura T. Adhesion and internalization of functionalized polystyrene latex nanoparticles toward the yeast Saccharomyces cerevisiae. Advan Pow Technol. 2014;25: 1394-1797.
35 Morais FA, Mello BA, Souza IA, Ponzi EA, Revoredo GA. Polymers a base methylmetacrylate. Importance in dentistry. Int J Dentistry. 2007;6(2): 63-66.
36 Xie J, Lee S, Chen X. Nanoparticle-based theranostic agents. *Adv Drug Deliv Rev*.2010;62: 1064-1079.
37 Ashemed N, Fessi H, Elaissari A. Theranostic applications of nanoparticles in cancer. *Drug Discov Today*. 2012;17: 928-934.