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Phagocytosis of PLGA Microparticles in Rat Peritoneal Exudate Cells: A Time-Dependent Study

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Abstract: With the purpose of enhancing the efficacy of microparticle-encapsulated therapeutic agents, in this study we evaluated the phagocytic ability of rat peritoneal exudate cells and the preferential location of poly(D,L-lactide-co-glycolic acid) (PLGA) microparticles inside these cells. The microparticles used were produced by a solvent evaporation method and were characterized by dynamic light scattering (DLS), transmission electron microscopy (TEM), and scanning electron microscopy (SEM). Size distribution analysis using DLS and SEM showed that the particles were spherical, with diameters falling between 0.5 and 1.5 μm. Results from cell adhesion by SEM assay, indicated that the PLGA microparticles are not toxic to cells and do not cause any distinct damage to them as confirmed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. Among the large variety of cell populations found in the peritoneal exudates (neutrophils, eosinophils, monocytes, and macrophages), TEM showed that only the latter phagocytosed PLGA microparticles, in a time-dependent manner. The results obtained indicate that the microparticles studied show merits as possible carriers of drugs for intracellular delivery.

Key words: microparticles, drug delivery, peritoneal exudate cells, macrophage, TEM, SEM

Introduction

In recent years, special interest has been focused on the development of drug delivery systems (DDS), prepared from polyester poly(D,L-lactide-co-glycolic acid) (PLGA) (Jain, 2000; Tunçay et al., 2000; Fu et al., 2002; Konan et al., 2003; Panyam & Labhasetwar, 2003; Panyam et al., 2003; Kumar et al., 2004). The synthetic biodegradable system used in this study consisted of PLGA microparticles, which have gained considerable interest due to properties like lack of antigenic action and the capacity to encapsulate both hydrophilic or lipophilic drugs (Kumar et al., 2004). This DDS has been investigated not only in the light of its controlled drug-releasing capacity, but also for its ability to reach its target cells, decreasing undesirable side effects and increasing the therapeutic ones (Okada & Toguchi, 1995). Aliphatic polyesters such as polylactide and its copolymers with glycolic acid have received considerable interest because they are biodegradable and biocompatible synthetic polymers that degrade to lactic ($C_3H_6O_3$) and glycolic ($C_2H_4O_3$) acids. Such polymers become degraded *in vivo* by hydrolysis of their ester linkages, a process that is acid, base, or enzyme catalyzed. Their breakdown products, CO_2 and H_2O, are easily eliminated (Dunne et al., 2000). These microparticles could offer a number of advantages over other delivery systems because: (a) they maintain their chemicophysical characteristics unaltered for long time periods, allowing extended storage; (b) depending on their composition, they can be administered through different modes of delivery (oral, intramuscular, or subcutaneous); and (c) they are suitable for industrial production (Lemoine et al., 1998; Esposito et al., 1999). Aiming at a number of applications, it is important to study the kinetics of cellular and tissue uptake, intracellular distribution and retention, and *in vivo* biodistribution of these microparticles (Panyam & Labhasetwar, 2003). For example, to optimize drug therapy, it might be required to study the efficiency of particle localization in a particular cell population, organ, or specific tissue following local or systemic administration (Lamprecht et al., 2001). Similarly, it is necessary to study the effect of various microparticle formulation parameters and their physical properties (e.g., surface charge, particle size) on their uptake and distribution within various cellular compartments (e.g., endo-lysosomes, cytoplasm, nucleus, etc.). Understanding

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the intracellular and tissue distribution of microparticles is also useful to elucidate the mechanism of enhanced therapeutic efficacy of particle-encapsulated therapeutic agents (Panyam et al., 2003). In this study we describe the internalization process of PLGA microparticles by rat peritoneal exudate cells in the absence of any opsonization processes.

Material and Methods

Materials

PLGA (50:50, Mw 17 kDa) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma Chemical, Inc. (St. Louis, MO), Polyvinyl alcohol (PVA) (13–23 kDa, 87–89% hydrolyzed) was supplied by Aldrich (Milwaukee, WI). Analytical grade dichloromethane was supplied by Merck (Darmstadt, Germany). All other chemicals were of analytical grade and were used without further purification.

Preparation of Polymeric Microparticles

Microparticles (MP) were produced by the solvent evaporation procedure described by Gomes et al. (2005). Typically, the organic phase consisted of 0.1 g of 50:50 PLGA polymer, dissolved in 10 ml of dichloromethane. It was dropped into an aqueous homogeneous phase (100 ml of an aqueous phase containing 3% [w/v] of 88% hydrolyzed PVA as dispersing agent), under ice cooling, and with stirring at 13,500 rpm, during 3 min. Solvent evaporation was then carried out by gentle magnetic stirring at room temperature, usually for 3–5 h. Microparticles were recovered by centrifugation at 4°C for 5 min at 10,000 rpm, washed three times with distilled water at 10°C, and lyophilized.

Morphology of Microparticles: SEM Analysis

SEM was used to evaluate the shape and size of PLGA microparticles. Samples were washed with sterile distilled water, fixed in 2.5% (v:v) glutaraldehyde in water, for 2 h, again washed with water, dehydrated in a graded ethanol series, and critically point dried. Samples containing microparticles were mounted on aluminum stubs and, because of their lack of electrical conductivity, coated with 50 nm gold coating under an argon atmosphere. Microparticle diameters were measured using a ruler and their mean value found using the scale on the SEM. A Electronscan (Philips ESEM 2020) operating at 5 kV was used for these measurements in the traditional mode (SE detector).

Particle Size and Surface Charge (Zeta Potential)

Surface charge and size of the microparticles were determined by photon correlation spectroscopy, using quasi-elastic light scattering equipment (Zetasizer 3000; Malvern Instrument, Worcestershire, UK, with a 10-mW He-Ne laser beam at 633 nm wavelength and 25°C and a scattering angle of 90°). A dilute suspension (1.0 mg/ml) of microparticles was prepared in doubly distilled water and sonicated in an ice bath for 30 s and subjected to particle size analysis. Zeta potential of microparticles in 0.1 mM Hank's buffer, pH 7.4 (1.0 mg/ml), was determined using ZetaPlus^{TM} in the zeta potential analysis mode.

Residual PVA Content

The residual amount of PVA associated with microparticles was determined by a colorimetric method (Sahoo et al., 2002). A calibration curve was prepared by dissolving PVA (2–10 mg) in 2 ml of 0.5 M NaOH for 15 min at 60°C. Samples were then neutralized with 900 μL of 1 M HCl and their volumes adjusted to 5 ml with distilled water. To each sample, 3 ml of a 0.65 M solution of boric acid, 0.5 ml of I_2/KI (0.05/0.15 M) solution, and 1.5 ml of distilled water were added. Finally, the visible spectra absorbance of the samples was measured at 690 nm (UV-1601PC UV-Vis Spectrophotometer, Shimadzu Scientific Instruments) after 15 min incubation (Figure 4, below). The lyophilized PLGA microparticles (2 mg) were solubilized in the same way to determine the residual PVA content. The values of absorbance of PVA obtained after microparticle dissolution was used to determine the concentration of residual PVA. This procedure was performed in triplicate.

MTT Assay

Cell viability was measured using a spectrophotometric MTT assay. To cells preincubated with microparticles of PLGA (0–1 mg/ml) for 2 h was added a MTT solution (150 μl per well), containing 4 mg per ml of the MTT reagent. The 24 well-containing plates used were incubated for 3 h at 37°C in a CO_2 incubator. The cells were then solubilized with an organic solvent (isopropanol) and the released solubilized formazen reagent measured spectrophotometrically. The absorbance at 570 nm was measured with a UV-1201 Shimadzu spectrophotometer and the percentage of vital cells calculated in relation to control cells incubated in the absence of microparticles.

Microparticles in Rat Peritoneal Exudate Cells: TEM Analysis

Time dependency studies were performed to evaluate the phagocyte ability of the peritoneal exudate cells from male Wistar rats weighing on an average 150.0 g ($n = 6$). Animals were anesthetized using a mixture of halothane, N_2O, and O_2, according to National Institutes of Health guidelines for the care and use of laboratory animals (NIH publication 85–23 Rev. 1985). After shaving and disinfection, 20 ml of PBS buffer were injected intraperitonealy into each rat; following a 5-min abdominal massage to free adherent macrophages, the PBS cell suspension was withdrawn with a syringe through a small incision made in the abdominal

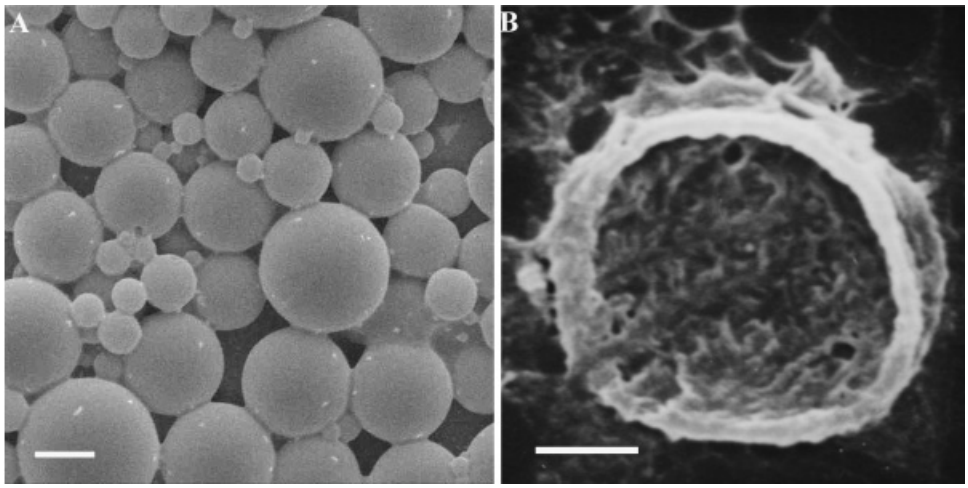

Figure 1. **A**: Morphology of the external surface of microparticles prepared by the solvent evaporation method. **B**: Idem of the inner surface after microparticle cross section. Magnification 15,000× and 50,000× by SEM. Bar = 0.1 μm and 0.5 μm.

wall. The peritoneal lavage fluid was centrifuged at 400×g for 10 min and the cell pellet resuspended in PBS buffer and divided into four aliquots of 2 ml each. Time-dependent assays were done incubating samples with PLGA microparticles (300 μg/ml) for 15, 30, and 120 min, respectively. Negative controls were incubated for 120 min, without microparticles. Following incubation, cells were washed twice with PBS solution. Possible untoward effects of fixative agents, such as 3.0% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4), 2.5% glutaraldehyde in 0.1 M synn collidine buffer (pH 7.4), and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 h at 25°C each, were evaluated in this study.

For TEM studies the samples were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 h at 25°C. Fixed cells were then postfixed in 1% osmium tetroxide in the same buffer for 1 h, dehydrated in a graded acetone series, and embedded in epoxy resin (Embed 812, Electron Microscopy Sciences, Hatfield, PA). Ultrathin sections were contrasted with alcoholic 2% uranyl acetate and 5% lead citrate. Ultrastructural examination was performed under a transmission electron microscope (Philips CM 100).

Results and Discussion

Microparticle Morphology

The morphology and size of the MP were determined by SEM. MP produced by solvent evaporation were spherical and possessed a smooth external surface (Fig. 1A). Figure 1B shows the inner surface of a cross-sectioned PLGA microparticle, demonstrating it to be a microcapsule (Ahsan et al., 2002).

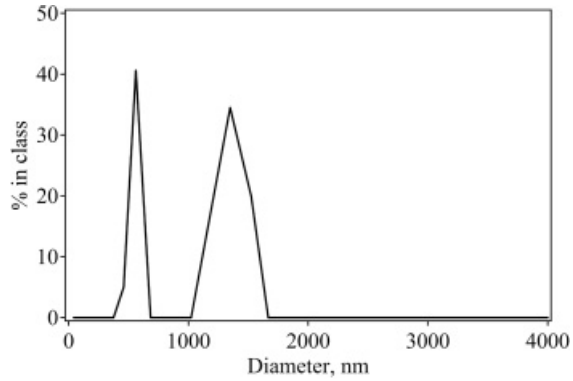

Figure 2. Size distribution of PLGA microparticles by dynamic light scattering.

Particle Size and Surface Charge (Zeta Potential)

Dynamic light scattering (DLS), demonstrated the MP analyzed to present a bimodal size distribution, their mean particle size varying between 0.5 and 1.5 μm (Fig. 2). Recent studies with nanoparticles in the biomedical and biotechnological areas showed their particle size to range between 10 and 1000 nm, acceptable for intravenous injection (Kreuter, 2004). The inconvenience of administering particles with a diameter of up to 4 μm seems to be their inefficiency as a DDS, because of possible accumulation in the lung capillaries and removal by the reticulo endothelial system (Jeon et al., 2000). Our modification of the solvent evaporation technique renders it possible to prepare smaller particles of $<$2.0 μm, useful as a DDS.

Colloidal stability was analyzed by measuring the zeta potential of the microparticles (Fig. 3). PLGA particles were

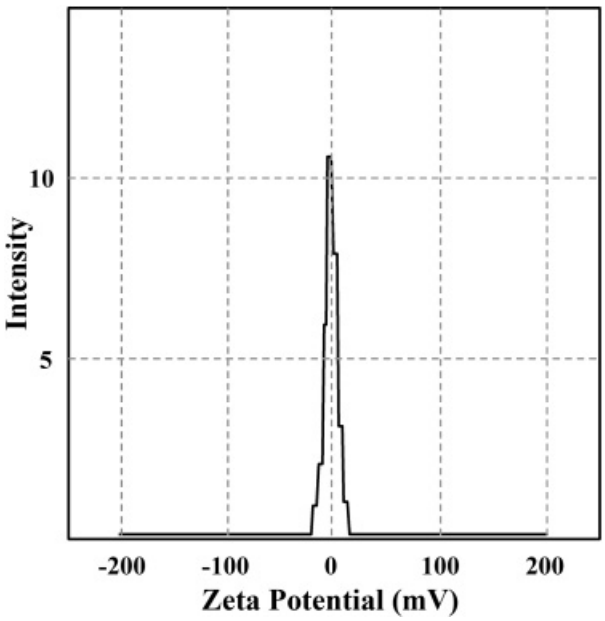

Figure 3. Zeta potential of PLGA microparticle's surface.

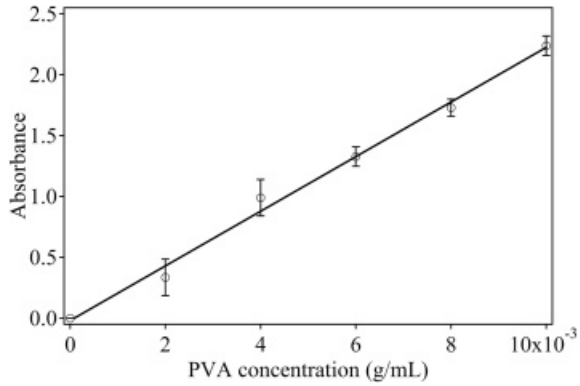

Figure 4. Calibration curve used to determine residual content of PVA on PLGA microparticles.

negatively charged (−3.2 mV at pH 7.4), a value normally considered to be associated with a stable colloid nature (Ruan & Feng, 2003). The negative zeta potential can be explained by residual PVA still present on the particle's surface even after three washings and which affects the number of carboxylate group endings (Lacasse et al., 1998; Kumar et al., 2004).

Residual PVA Content

The percentage of residual PVA was in the range of 1.6%–3.4% (w/w) (Fig. 4). According to Sahoo et al. (2002), the fraction of PVA that remains associated with the surface of the particles affects their physical and cellular uptake properties. Particle hydrophobicity decreases with the amount of associated PVA reducing their cellular uptake (Sahoo et al., 2002). In our work, PLGA microparticles were washed three times with PBS buffer to remove the excess PVA, resulting in improved particle recognition by macrophages.

Incubation Process of MP with Peritoneal Exudates Cells

Following incubation of MP with peritoneal exudate cells for 15, 30, and 120 min, respectively, the efficiency of phagocytosis (adhesion or phagocytosis) was evaluated by SEM. Figure 5A shows a control cell (without MP), Figure 5B a cell incubated with MP at zero time, and Figure 5C peritoneal exudate cells incubated with MP for 30 min. After this period, particles had already adhered to the cell surface. Figure 5D shows that after 120 min, the particles had been completely phagocytized by the cells, appearing as extensions of the cell surface. This result furnished the minimum time period required for a large majority of the MP to be engulfed by phagocytosis by the peritoneal fluid exudate cells.

MTT Assay

Microparticle toxicity to peritoneal exudate cells was investigated by exposing macrophages to increasing amounts of the microparticles. Viability evaluated by the MTT test was not affected in macrophages exposed to 0.1 to 1.0 mg/ml of MP, respectively (Fig. 6; Artursson et al., 1987). This indicates that the PLGA microparticles were not toxic to the cells and could be used as a DDS.

Microparticles in Rat Peritoneal Exudates Cells: TEM Analysis

Biocompatibility studies using microspheres *in vitro* generally employ a cell culture model (Panyam et al., 2003). In the present work we used peritoneal exudate cells from live male Wistar rats. Ultrastructural examination was performed by TEM (Fig. 7A, control). The experiments demonstrated that microparticles entered macrophages through an apparently conventional form of phagocytosis, in which phagocyte pseudopods moved circumferentially around the microparticles until fusing at their distal tips. Microsphere contour boundaries were clearly observed without the need for any specific electron-dense marker (Fig. 7B,D, arrow).

Our results indicate that in a time-dependent manner, following 15 min of incubation phagocytosis begins (Fig. 7B), followed by internalization of the particles for 30 min (Fig. 7C). After 120 min, PLGA microparticles of different sizes were located inside the macrophages (Fig. 7D). The experiment also demonstrated that almost all MP were phagocytosed within 120 min. We believe that the TEM technique is more sensitive for assessing newly ingested microparticles, because obviously, the number of microparticles phagocytosed per macrophage increases with incu-

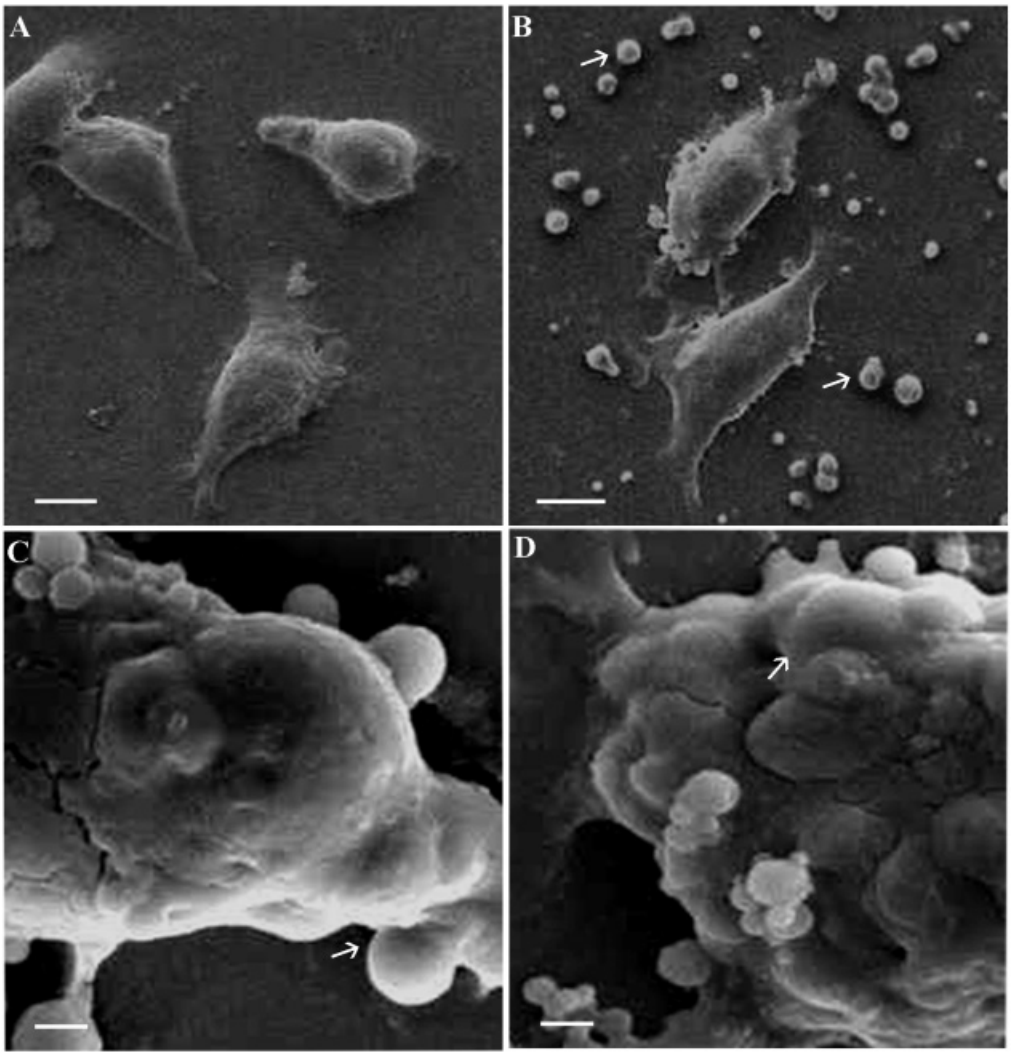

Figure 5. Scanning electron micrograph of macrophages exposed to microparticles (arrow). **A:** Control cell (without MP); **B:** Cell incubated with MP at zero time; **C:** Peritoneal exudate cells incubated with MP for 30 minutes; **D:** Cell incubated with MP for two hours of incubation. Bar = 1.3 μm.

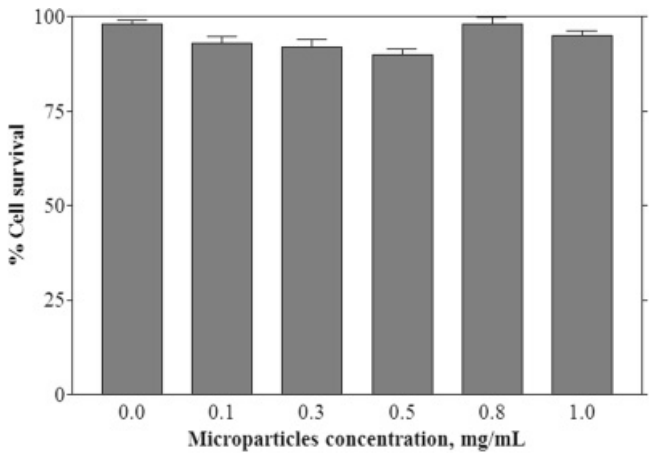

Figure 6. Effect of PLGA microparticles on cells of rat peritoneal fluid exudate measured by a MTT assay.

bation time. It has been demonstrated that particle uptake by phagocytic cells is largely affected by the size and surface properties of the particles (Tabata & Ikada, 1988; Moghimi et al., 2001; Ahsan et al., 2002).

The external surface of PLGA microparticles possesses hydroxyl groups of PVA molecules attached to PLGA acetyl groups via hydrophobic links (Murakami et al., 1999). The reaction of PVA cross-linked by glutaraldehyde was observed by treating a PVA membrane surface with a specified amount of a dialdehyde such as glutaraldehyde (Castelli et al., 2000). Among the four fixative solutions used here, the best results for the preservation of the microparticles were the use of the combination of glutaraldehyde with cacodylate buffer (pH = 7.4), as illustrated in Figure 7. The use of glutaraldehyde in this buffer instead of the PBS buffer offers a greater advantage due to the reduction of polymerization of glutaraldehyde with an increase of its

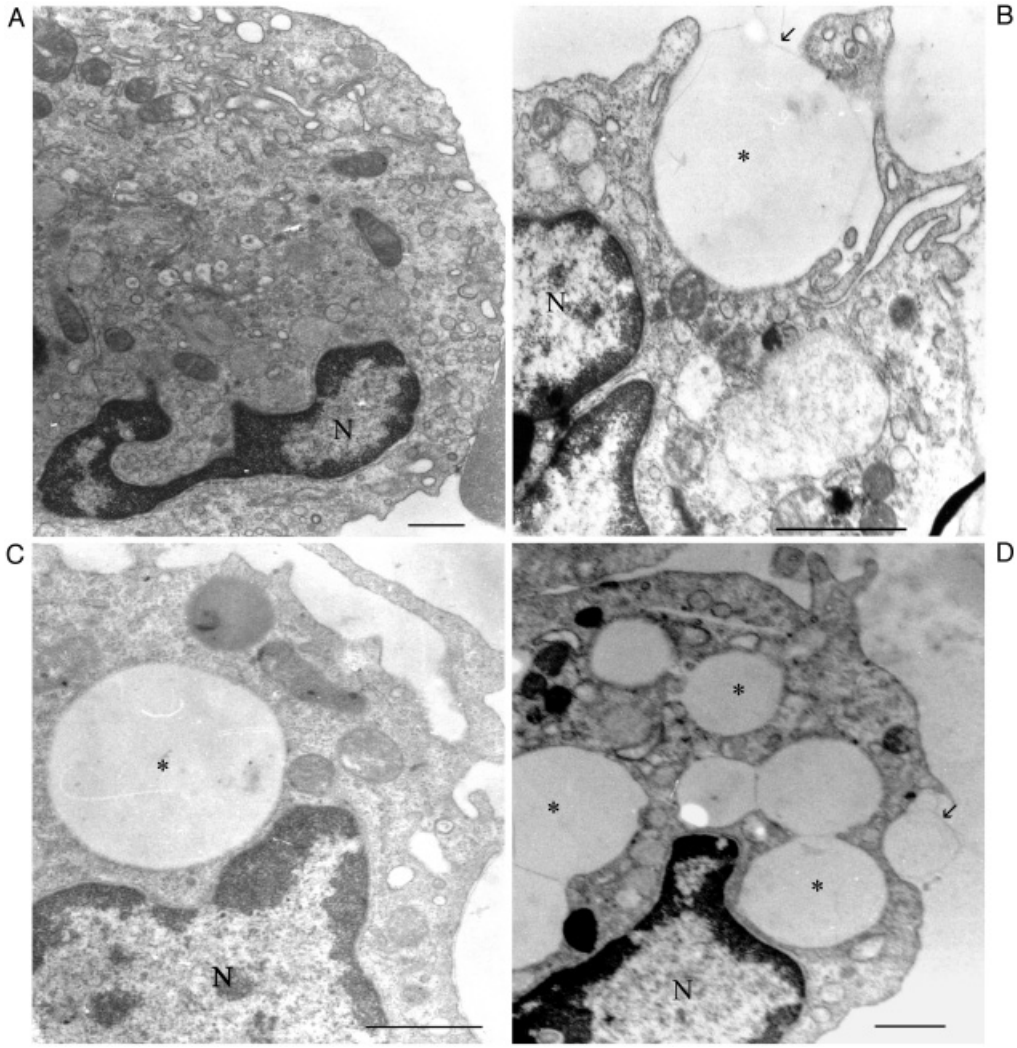

Figure 7. **A**: Exudate macrophage cell without microparticles (control). N: nucleus. **B**: PLGA microparticles (indicated by asterisks) interacting with the cell membrane in the process of phagocytosis after 15 min of incubation. **C**: Internalization of PLGA microparticles. Phagosome after 30 min of incubation. **D**: Heterogeneous population of PLGA microparticles (asterisks) phagocytosed by macrophages after 120 min of incubation. Bars = 0.5 μm.

fixative activity (Hayat, 1981). This feature enhanced the visualization of the microparticles by TEM.

Conclusion

Our studies showed good preservation of PLGA microparticles, achieved by the use of a combination of glutaraldehyde and cacodylate buffer. They demonstrate that the uptake of the microparticles by peritoneal exudate phagocytic cells exhibits a time dependence for particle attachment and internalization, and that this process was observed only for macrophage cells.

Acknowledgments

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