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Polymeric Nanogels:

Nanocarriers For Drug Delivery Application.

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The application of nanotechnology to medicine has enabled the development of functionalised nanoparticles that, acting as carriers, can be loaded with drugs or genetic material to be released with a controlled mechanism in specific districts of the organism. Even though nanomedicine is a relative new branch of science, many type of nanocarriers for drug delivery have been developed over the past 30 years, such as liposomes, dendrimers, quantum dots, solid lipid nanoparticles, viruses and virus-like nanoparticles as well as a wide var iety of polymeric nanoparticles. Among these last, in our opinion, nanogels deserve a special attention.
Nanogels are nanoscalar polymer networks, with a tendency to imbibe water when placed in an aqueous environment. Their affinity to aqueous solutions, superior colloidal stability, inertness in the blood stream and the internal aqueous environment, suitable for bulky drugs incorporation, make them ideal candidates for uptake and delivery of proteins, peptides, and other biological compounds. We have synthesised different variants of poly(N-vinyl-pyrrolidone)-based nanogels and demonstrated the absence of cell toxicity, which encourage further development of these materials as smart delivery systems.
In particular, in this work we demonstrate the capability of these nanogels to bypass the cell plasma membrane by following their localization in cell cultures as function of the time. We have analyzed this process by both confocal microscopy and a spectrofluorimetric approach. Results show nanoparticles preferential localization on cell surface, inside the cell and again back in the cell culture medium at different times. Ongoing experimentation is now aimed to the loading of nanocarries with biomolecules involved in a specific substrate recognition function. This approach, if proved successful, may have a real impact in nanomedicine.

1. Introduction

The development of nanoparticles for delivery of therapeutic agents has introduced new opportunities for the improvement of medical care and, even though nanomedicine is a relative new branch of science, many type of nanocarriers for drug delivery have been proposed over the past 30 years, such as: liposomes, dendrimers, quantum dots, solid lipid nanoparticles, viruses and virus-like nanoparticles, as well as a wide variety of polymeric nanoparticles.

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The motivation of the growing interest for these systems is to find alternative and more effective procedures than conventional therapies in the pharmacological treatment of various diseases.
Among the many micellar aggregates and their mediated systems, polymeric nanoparticles have been designed to successfully encapsulate drugs in order to target cells and avoid drug degradation and toxicity as well as to improve drug efficacy (Allermann et al., 1993; Couvreur et al., 2002).
Functionalized nanoparticles can be very useful for various diseases like cancer, a leading cause of morbidity and mortality worldwide, or in diseases of the central nervous system, whose treatment is very difficult because the brain is not directly accessible to intravenously (i.v.) administered drugs owing to the presence of the blood–brain barrier (BBB) (Patel et al., 2009). The development of drug delivery systems able to diffuse into the central nervous system (CNS) or to localize preferentially, if not exclusively, at the tumor site represents one of the main fields of interest in modern pharmaceutical research.
In our opinion, in the realm of polymeric nanocarriers, nanogels deserve a special consideration. Several investigations have shown that nanogels can be used efficiently as colloidal supramolecular devices for the delivery of oligonucleotides into the brain (Vinogradov S.V. et al., 2004), because of their favorable physicochemical properties, such as their narrow size distribution (Soni S. et al., 2006).. The goal of this strategy resides in the absence of adverse toxic effects from these devices after *in vivo* administration (Vinogradov et al., 2004).
Nanogels are nanoscalar polymer networks, with a tendency to imbibe water when placed in an aqueous environment. Their affinity to aqueous solutions, superior colloidal stability, inertness in the blood stream and the internal aqueous environment, suitable for bulky drugs incorporation, make them ideal candidates for uptake and delivery of proteins, peptides, and other biological compounds.
A typical advantage of these hydrogel nanoparticles, respect to the classic nanoparticles, is the possibility of obtaining an elevated degree of encapsulation and offering an ideal tridimensional microenvironment for many macromolecule types. Due to their molecular size, ranging between 100-700 nm, nanogels can escape renal clearance and have prolonged serum half-life period (Wilk et al., 2009). Often they cannot penetrate the endothelial junctions of normal blood vessels. But vascular endothelium in pathological sites (solid tumors, inflammation tissues and infracted areas) is discontinuous with large fenestrations of 200-780 nm, which allow the nanoparticle passage (Gaumet et al., 2008).
Nanoparticles-based controlled-release systems can follow two distinct approaches: a "passive targeting", where the nanoparticles loaded with biological molecules show a preferential release in the specific district where they were inoculated or where they accumulate in relation of their size and/or surface charge or other physico-chemical properties. In this case, a non-specific uptake of the nanoparticles in circulation is still present in healthy organs.
The second strategy is the "active targeting": in this case the nanoparticles are functionalized with molecules able to recognize and bind specific receptors over-expressed in target cells, such as cancer cells in which the site-specific accumulation becomes maximum. This mechanism is able to improve the therapeutic strategy, limiting toxicity around to the addressed site, differently from e.g. traditional chemotherapy where there is an aspecific drug distribution.
Controlled release of drugs encapsulated into the functionalized-nanoparticles can also be induced by external stimuli, for example by application of ultrasounds or an increase of temperature, etc. With an active targeting in mind, we have synthesised and characterised different variants of amino-functionalised poly(N-vinyl-pyrrolidone)-based nanogels.
The amino groups are amenable of bioderivation with biologically relevant molecules for targeting purposes. We have demonstrated the absence of cell toxicity, which has encouraged further development of these materials toward the application for which they have been designed. In particular, in this work we show the capability of these nanogels to bypass the cell plasma membrane, following their localisation in the cells as function of the time.

2. Experimental

2.1 Materials

Nanogels of poly(N-vinyl-pyrrolidone)-co-aminopropylacrylamide(PVP-APMAM) were produced by pulsed electron irradiation of PVP-APMAM aqueous solutions with a Linac 10MeV accelerator. Irradiation was performed with Electronika, a 10 MeV linear accelerator at the ICHTJ of Warsaw (Poland), equipped with a conveyor belt that allows many vials in a tray to pass under the beam at a given speed, which was set to supply 40 kGy per pass. An integrated dose of 80 kGy was supplied with two passes under the beam. Temperature was always maintained between 4-10 °C. After synthesis, nanogels were. conjugated with fluorescein isothiocyanate (FITC) and extensively dialyzed prior to the use in cell cultures. Nanogel concentration in the aqueous dispersion is approximately 1 mg/ml.

The osteoblastic cell line MC3T3-E1 were obtained from a C57BL/6 mouse calvaria (Healt Protection Agency) and were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal calf serum (FCS; Euroclone, Celbar), 1 % antibiotic and 1 % glutamine (Euroclone, Celbar).

2.2 Cellular internalization studies: Confocal Analysis

The MC3T3-E1 were grown at a density of 5×10^3 cells/well into 12-well plates containing sterile glass coverslips in complete DMEM, for 24 h. Next, the cells were incubated with FTIC -labeled PVP-APMAM nanogels, which emit green fluorescence by FITC conjugation.

After different incubation times, respectively, 1 h, 3 h, 6 h, 8 h and 24 h, the cells were washed twice with PBS to remove nanoparticles not taken up by the cells, fixed with 3.7 % formaldehyde for 15 min, and again washed twice with PBS.

Afterwards, the cells were stained with ethidium bromide (1:1000) for 1 min at room temperature. The monitoring was carried out observing the green fluorescence within the cells by confocal microscopy (Olympus 1x70 with Melles Griot laser system).

2.3 Cellular internalization studies: Spettrofluorimetric Analysis

The MC3T3-E1 cells were seeded in a 96 well tissue plate at a density of 7×10^3 cells/well and grown for 24 h in DMEM complete medium. Then, the cells were incubated with PVP-APMAM-FITC (4ug/well) nanogels and the nanoparticles percent into the cells after several incubation periods (0; 30', 1 h, 3 h, 6 h, 24 h and 48 h) was calculated by spectrofluorimetric readings (excitation at 485 nm and emission at 538 nm) using Spectra Max Gemini EM-500 (Molecular Devices) developed by Soft Max Pro 5.2 software.

In particular, for each incubation period and each sample (represented by 12 wells), the total fluorescence, as obtained by the fluorescence reading from cells incubated in a complete medium added with the nanoparticles, was read. Also, the cell's conditional medium was moved in a new 96 well culture plate and the fluorescence present only in the medium was read.

After removing the medium from the cells, they were washed twice with PBS to remove the excess of nanoparticles that hadn’t internalized yet. The washes were collected in another 96 well culture plate and the residual fluorescence was also read. The fluorescence intensity from the culture medium, the cells and washings was checked to be equal to the initial total fluorescence.

3. Results and Discussion

3.1 Image Analysis Of Nanogels-Cells Interaction

In order to investigate nanogels interaction with cells, we tested PVP-APMAM-FITC nanoparticles in presence of MC3T3-E1 cells for different incubation periods and we followed their movement inside and through the cells.

As shown in Figure 1, after 1 h of incubation, nanogels are distributed around the cells’ surface. Whereas, after 3 h of incubation, it was noted their penetration into the cytoplasm compartment. A similar distribution can be observed also in samples incubated for 6h,, and then the nanogels concentration begins to decreases over time.

Probably, the nanogels are excreted as waste products in the extracellular environment, although a degradation by the lysosomal cell system of the PVP-based nanoparticle itself or of the link with the fluorescent label cannot be excluded.

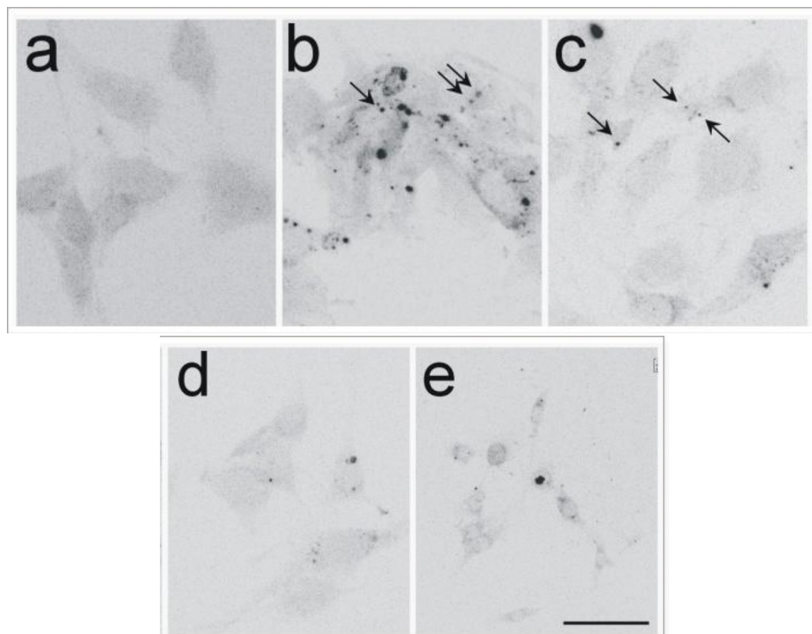

Figure 1: Nanogels-cell interaction : PVP-APMAM-FITC was incubated with MC3T3-E1 cells for different time. The localization was valued after 1 (a); 3(b); 6 (c); 8 (d) and 24 (e) hours respectively. Images, obtained by confocal microscopy observations, show the fluorescent nanoparticles. Arrows indicate the nanoparticles into the cells. Bar= 10 μm

3.2 Path of nanoparticles inside the cells

Nanoparticles distribution was quantitatively estimated, by spectrofluorimetric measurements, at different time. For this purpose, FITC-conjugated PVP-APMAM nanoparticles were incubated with MC3T3-E1 cells.

Through spectrofluorimetric readings, for each sample, the total fluorescence (in the cells and in the medium), the amount of fluorescence inside the cells and of the fluorescence present in the waste wash were measured.

As shown in the Figure 2, at 0 time a high percent of fluorescence was present in the culture medium (86 %) and in the wash (14 %), indicating that the nanoparticles hadn't interacted with the cells yet. After 30 min of incubation, the fluorescence present inside the cells was about 3 % of the total, indicating the onset of uptake. We can suppose that this could be due to the addressing of nanoparticles in direction of cell endocytotic pathway, that could be associated to the typical clathrin-coated vesicles transport system.

On the other hand, a decrease of the medium fluorescence about 72 % was detected and an increase in the wash (25 %) is present. These data suggest that the excess of nanoparticles that are not internalized, are then removed through the wash, just as in the early times.

After 3 h, the cells-fluorescence was increased up to 5 %, while decreasing both in the wash and culture medium.

At 6 h fluorescence inside to the cell reached the maximum value (7 %) and decreased until to 3 % in 48 h. The rest of fluorescence was mainly concentrated into the culture medium (about 80 %), and to a lesser extent, in the wash (17 %); suggesting a nanoparticles extrusion mechanism through the exocytotic path.

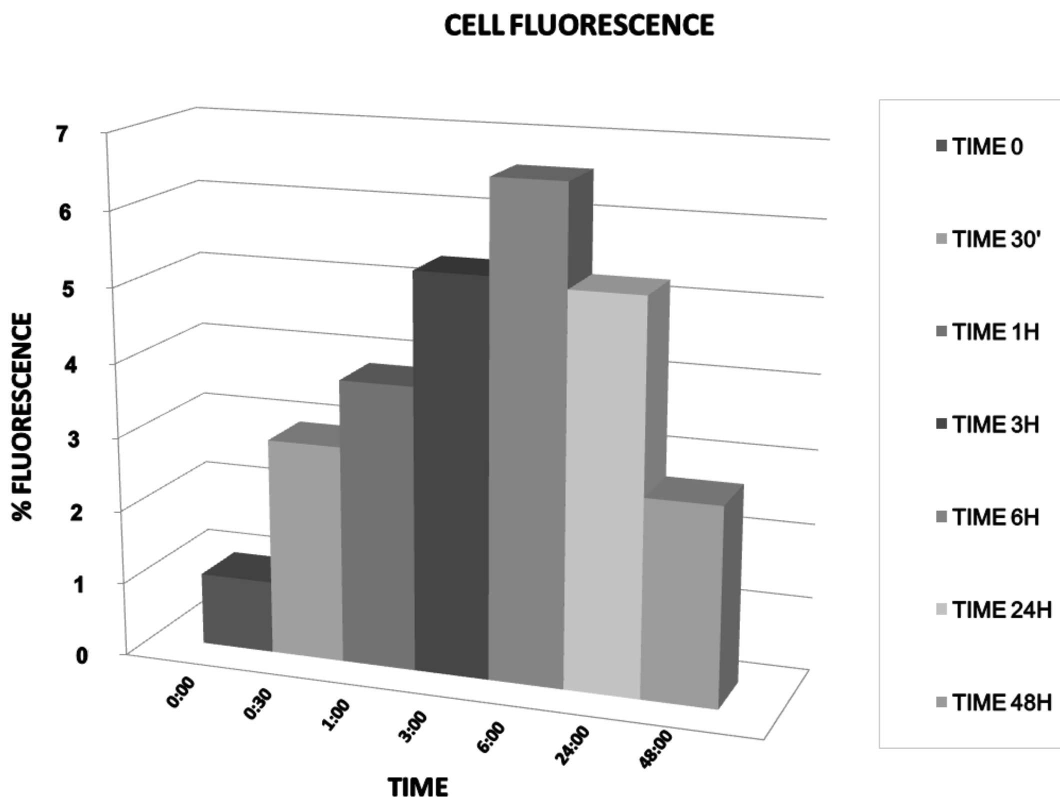

Figure 2: Path of nanoparticles through the cell compartments. PVP-FITC was incubated with MC3T3-E1 cells for different time. The nanoparticles localization was valued by spettrofluorimetric analysis. The graph shows the percentage of cells fluorescence. Standard deviation was about 2% in each samples.

4. Conclusions

Cellular-internalization experiments showed that our nanoparticles exhibited a significantly enhanced cellular accumulation, without causing observable cytotoxic processes; according to previous results obtained in cytocompatibility and toxicity test (data not shown).

This study, although preliminary, offers an exciting prospect in use the generated nanoparticles as potential drug delivery carriers thanks to their capability of using a physiological pathway to come through the cell compartments. Therefore, it encourages to further progress in the design, manufacture and evaluation of advanced nanogels with embeded cell-target specific recognition functions.

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