# *In vivo* biodistribution of carboxymethylchitosan/poly(amidoamine) dendrimer nanoparticles in rats

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## Abstract

Carboxymethylchitosan/poly(amidoamine) (CMCht/PAMAM) dendrimer nanoparticles, comprised of a PAMAM dendrimer core grafted with chains of CMCht, have recently been proposed for intracellular drug delivery. In previous reports, these nanoparticles had improved cytotoxicity compared with traditional dendrimers. In this study, the short-term *in vivo* biodistribution of fluorescein isothiocyanate (FITC)-labeled CMCht/PAMAM dendrimer nanoparticles after intravenous (IV) injections in Wistar Han rats was determined. The brain, liver, kidney, and lung were collected at 24, 48, and 72 h after injection and stained with phalloidin–tetramethylrhodamine isothiocyanate (TRITC, red) and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, blue)[AQ: 1] to trace the nanoparticles within these tissues. The liver, kidney, and lung were also stained for hematoxylin and eosin to assess any morphological alterations of these organs. CMCht/PAMAM dendrimer nanoparticles were observed within the vascular space and parenchyma of liver, kidney, and lung and in the choroid plexus, after each injection period. No particles were observed in the brain parenchyma, nor any apparent deleterious histological changes were observed within these organs. The CMCht/PAMAM dendrimer nanoparticles were stable in circulation for a period of up to 72 h and targeted the main organs of the systems by internalizing by the cells present in their parenchyma.[AQ: 2] These results provide positive indicators to their potential use in the future as intracellular drug delivery systems.

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## Introduction

Dendrimers are a class of highly branched synthetic polymers that form spherical macromolecules that may be synthesized to a specific physical size in a highly reproducible manner.[1] Typically, the synthesis route occurs in a layer-by-layer fashion ('generations') around a core unit, resulting in high level of control over size, branching points, and surface functionality.[2] The unique features of the dendrimers and its derivatives make them very attractive for the transport of macromolecules within the space between the layers (by encapsulation) or, alternatively, through linkage to the terminal groups located on the surface.[3,4] Given the polyvalent features of the dendrimers, their application has ranged from gene transfection agents,[5] targeted drug delivery systems,[6,7] or contrast carriers for bioimaging.[8,9]

Although the potential application of these agents is very promising for intracellular drug delivery, the *in vivo* toxicity profile of these systems is a major concern. In general, cationic dendrimers are hemolytic and cytotoxic, both depending on molecular weight (generation) and number of terminal groups on the surface.[10] Thus, when the size of the dendrimer is increased for larger loading capacity, the cytotoxicity increases. This problem can be mitigated by the manipulation of the surface functional groups, which render these nanoparticles with more amenable characteristics for biological applications.

Recently, we proposed an alternative type of dendrimer-based nanoparticles, consisting of a poly(amidoamine) (PAMAM) dendrimer with carboxymethylchitosan (CMCht) chains on the surface, denominated as 'CMCht/PAMAM dendrimer nanoparticles'.[12] By doing so, it was possible to maintain the amenable cell internalization properties of PAMAM dendrimers, while increasing the drug-loading capacity with CMCht, without inducing any cytotoxic effects on the targets cells.[12] Moreover, since CMCht is a biodegradable biomaterial, it should be possible to tune its degradation according to the properties of the cell/tissue undergoing a therapeutic action. When compared with the traditional dendrimer-based systems, these CMCht/PAMAM dendrimer nanoparticles encapsulated greater drug loads. These 26-nm CMCht/PAMAM dendrimer nanoparticles had a favorable toxicological profile *in vitro* when cultured with L929 fibroblasts, even when compared with nonmodified dendrimeric systems. They were also included in the development of novel strategies for differentiating rat bone marrow stromal cells toward the osteogenic lineage for future bone regeneration.[12] These systems may potentially be used for intracellular drug delivery within the central nervous system (CNS), as all CNS cell types internalize them.[13]

The aim of this work was to assess the short-term *in vivo* biodistribution of CMCht/PAMAM dendrimer nanoparticles injected intravenously (IV) in male Wistar Han adult rats. The *in vivo* internalization of the nanoparticles by different cell types was also investigated by using a fluorescein isothiocyanate (FITC) probe. The liver, kidney, and lung were also stained for hematoxylin and eosin in order to assess possible alterations in the morphology of these organs.

## Materials and methods

### CMCht/PAMAM dendrimer nanoparticles synthesis

The synthesis route was performed as previously described by Oliveira et al.[12] CMCht was synthesized by a chemical modification route of chitosan (Sigma, Germany) as described by Lian et al.[14] **[AQ: ]** In brief, medium-molecular-weight chitosan (20 g; Aldrich, St. Louis, MO) with particle sizes from

125 to 250 µm was dispersed in 200 mL of isopropyl alcohol, and 50 mL of 10 N aqueous sodium hydroxide was added with vigorous stirring under a $N_2$ atmosphere. After 2 h and 18 g of mono-chloroacetic acid solid was added, the reaction mixture was heated at 60°C for 1 h. Distilled water (17 mL) was added to the mixture, and pH was adjusted to 7.0 with dilute acetic acid. The product obtained was filtered and washed with anhydrous methanol and dried overnight at 60°C. The particles were then dialyzed in water for 5 days and freeze-dried (Telstar-Cryodos, Spain) at −80°C. The CMCht product was characterized for degree of deacetylation (DD) of 80% and degree of substitution (DS) of 47% by ^{1}H-NMR and Fourier transform infrared (FTIR) analyses.**[AQ: ]**

*Preparation of intermediates*. The generation of the PAMAM-carboxylic–terminated dendrimers (PAMAM-CT, G1.5) and PAMAM-methyl ester–terminated dendrimer were prepared as follows: PAMAM-CT (G1.5) was dissolved in ultrapure water and then 1 mol, per each mol of surface carboxylic group, of 1-ethyl-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC; Fluka, Slovakia) was added. The pH was maintained at 6.5, under agitation; after 30 min, ethylenediamine (EDA; Sigma, Germany) was added (1:1 molar ratio, EDA to EDC). After purification by dialysis, the PAMAM-methyl ester dendrimer was synthesized by adding methyl methacrylate (Fluka, Germany).

*Dendrimer nanoparticles*. The dendrimer nanoparticles were produced, with carboxylic-terminated groups, by mixing CMCht (100 mg) and the PAMAM-methyl ester dendrimer (50 mg) in a water/methanol solution for 72 h. The CMCht/PAMAM dendrimer nanoparticles were obtained by precipitation in a saturated sodium carbonate ($Na_2CO_3$) solution and cold acetone. The particles were collected by filtration, dispersed in ultrapure water for extensive dialysis, and freeze-dried. The CMCht/PAMAM dendrimer nanoparticles had a mean size ranging from 22.0 to 30.7 nm for a confidence level of 95%, and a confidence level of ±4.4 as reported.[13] **[AQ: 6]** In aqueous solution, the dendrimer nanoparticle sizes were from 45 ± 15 nm to 250 ± 100 nm.

*FITC labeling*. To trace the nanoparticles within the different tissue and organ compartments, CMCht/PAMAM dendrimer nanoparticles were labeled with FITC (Sigma, Germany).[12] Conjugates of CMCht/PAMAM-FITC were prepared by covalently bonding the CMCht amine with the isothiocyanate group of FITC (thiourea bond). A CMCht/PAMAM dendrimer nanoparticle solution was prepared by dissolving the nanoparticles in a carbonate/bicarbonate coupled buffer of pH 9.2 to form a 10 mg/mL of solution. FITC/dimethyl sulfoxide (DMSO, 50 µL) was added per 1 mL of CMCht/PAMAM dendrimer nanoparticle–buffered solution; the mixture was agitated for 8 h at 4°C in the dark. The FITC-labeled CMCht/PAMAM dendrimer nanoparticle solution was dialyzed (to remove the unbound FITC) and freeze-dried at −80°C. The labeling efficiency was investigated by reading the absorbance at 280 and 495 nm (UV–vis spectrophotometer ND-1000; NanoDrop Technologies, Wilmington, DE)**[AQ ]**, and the molar ratio of FITC:CMCht/PAMAM dendrimer nanoparticles was calculated using a standard curve.

## In vivo assays

Male Wistar Han adult rats (10 weeks old, Charles-River Laboratories, Wilmington, MA)**[AQ: 8]**, were housed (two per cage) under standard laboratory conditions (12 h light–dark cycles, at 22°C, relative humidity of 55%; free access to food and water). All procedures were carried out in accordance with European Union Directive 86/609/EEC and National Institutes of Health guidelines on animal care and experimentation.

To evaluate the biodistribution of these nanoparticles, the animals were randomly distributed in four groups: (1) control group, not submitted to any injection; (2) 'sham' group, injected IV with α-minimum essential medium (MEM)**[AQ ]** only; (3) 'low-dose' group, injected IV with 1 µg/g of FITC-labeled CMCht/PAMAM dendrimer nanoparticles in α-MEM; and (4) 'high-dose' group, injected IV with 10 µg/g of animal weight of FITC-labeled CMCht/PAMAM dendrimer nanoparticles in α-MEM. The injections were performed on the tail vein of each animal (500 µL/injection).

After 24, 48, and 72 h (*n* = 3 each time), the animals were sacrificed, and the brain, kidney, lung, and liver were collected, rapidly frozen by immersion in liquid nitrogen protected by OCT (Sakura-Finetek, Holland), **[AQ: ]** and stored at −20°C. To trace the nanoparticles in the tissues, the organs were cryosectioned (20 μm), and the slices stained for cytoskeleton using tetramethylrhodamine isothiocyanate (TRITC)–phalloidin (Sigma–Aldrich) that links to actin. The slices were fixed with 4% paraformaldehyde permeabilized with Triton-X 0.3%, further blocked with fetal bovine serum 1%, incubated with phalloidin (1:300), and finally stained with 4′,6-diamidino-2-phenylindole (DAPI, 1:2000) for nuclear labeling.

Since toxicity is a major issue with any new biomaterial application, the histology of liver, lung, and kidney was assessed to exclude the presence of any gross morphologic alteration triggered by the CMCht/PAMAM dendrimer nanoparticles. For this reason, portions of the liver, kidney, and lung were collected immediately after sacrifice, embedded in paraffin, and stained with hematoxylin and eosin.

## Flow cytometry

Cerebrospinal fluid (CSF) was collected, when the animals were sacrificed, and analyzed by flow cytometry to evaluate the presence of FITC-labeled CMCht/PAMAM dendrimer nanoparticles. As control, we used CSF from Group 2. The samples were analyzed on a FACSCalibur cytometer, and the resulting data were processed using CellQuest software (BD Biosciences, Franklin Lakes, NJ ). **[AQ ]**

## Results and discussion

All the organs analyzed revealed the presence of FITC-labeled CMCht/PAMAM dendrimer nanoparticles (Figure 1) at 24, 48, and 72 h upon IV injection in the dose of 10 μg/g of animal weight. In contrast, the concentration of 1 μg/g (data not shown) was insufficient for an efficient tracing and evaluation of the biodistribution of the nanoparticles, as only residual levels of green fluorescence were observed in this group.

Liver and lung are important components of the reticuloendothelial system (RES) and thus are involved in the clearance of macromolecules.[15] Additionally, due to their rich vasculature, these organs are likely targets for the location of the dendrimer nanoparticles after IV injection. FITC-labeled CMCht/PAMAM dendrimer nanoparticles were observed in the liver (Figure 1), both in the vascular space and in the parenchyma in all time points analyzed. Moreover, it was also possible to observe *in vivo*, the internalization of the CMCht/PAMAM dendrimer nanoparticles (Figure 1) by cells. The presence of nanoparticles in the liver parenchyma indicates their ability to cross the endothelium. Of notice, the biodistribution of these nanoparticles within the liver parenchyma is not homogeneous. In the lung, the FITC-labeled CMCht/PAMAM dendrimer nanoparticles were predominantly observed in the alveolar wall and alveolar space (Figure 1). These observations reinforce the ability of the nanoparticles to cross the endothelium and reach the extravascular space.

Regarding the kidney, the FITC-labeled CMCht/PAMAM dendrimer nanoparticles seem to be present both in the glomerulous and renal tubules. The kidney plays an important role in the clearance of macromolecules circulating in the bloodstream.[15] As revised by Aillon et al.,[16] lower generations of PAMAM–gadolinium used as contrast agents (diameter 10 nm) were excreted *via* kidney, and the renal elimination rate decreased as high RES uptake increased with increasing dendrimer size above 10 nm. In this sense, the presence of these nanoparticles within the kidney may result from the excretion of smaller nanoparticles present in circulation while the bigger ones may be degraded by the RES or excreted in bilis. Alternatively, accumulation of nanoparticles may occur in kidney due to molecular interactions and electrostatic forces, even though the injected dose is probable insufficient to provoke such effect. The analysis of urine samples and the performance of
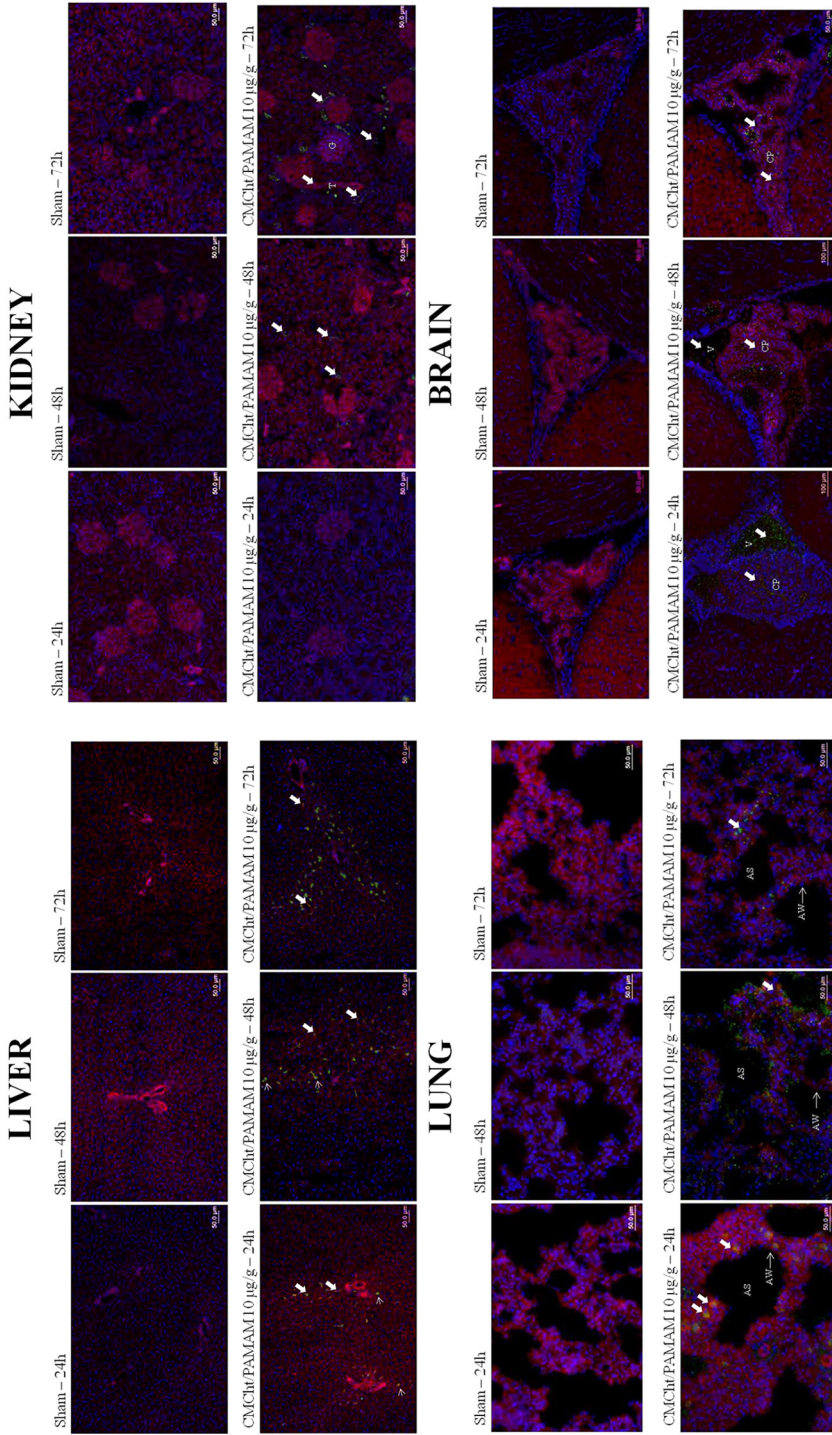
**Figure 1.** Fluorescence micrographs of kidney, liver, lung, and brain. These organs were cryosectioned (20 μm) and stained with phalloidin–TRITC for actin (red) and DAPI for nucleus (blue). It can be observed that the FITC-labeled CMCht/PAMAM dendrimer nanoparticles (green) were present in all organs studied after 24, 48, and 72 h of incubation when the concentration of 10 μg/g was used. Controls and shams: Moreover, there are evidences that CMCht/PAMAM dendrimer nanoparticles were able to cross some of the biologic membranes given that they can be seen in ventricular space in brain and alveolar space in lung (AS, alveolar space; AW, alveolar wall; G, glomerulous; T, tubules; CP, choroid plexus; V, ventricle)
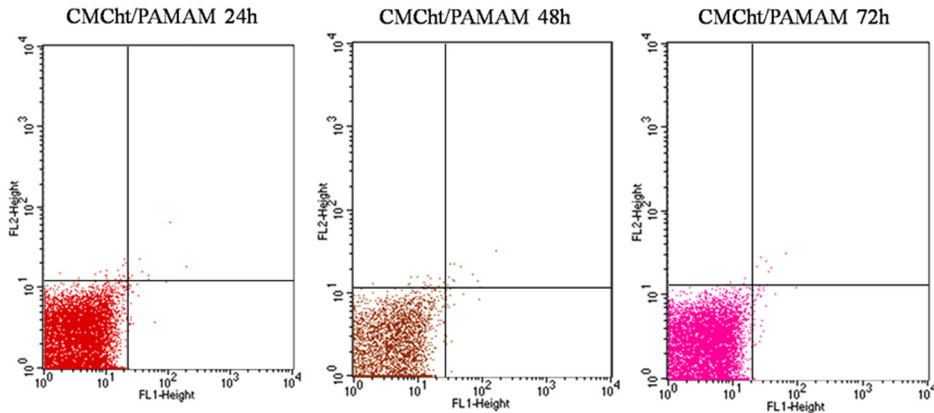
**Figure 2.** ▭ plot analysis gathered by flow cytometry of CSF obtained from animals injected with 10 μg/g of CMCht/PAMAM IV after 24, 48, and 72 h. Results revealed the presence of FITC-labeled CMCht/PAMAM dendrimer nanoparticles in CSF of animals within this group, with 0.11% ± 0.03%, 0.16% ± 0.02%, and 0.27% ± 0.05% of FITC-labeled nanoparticles after 24, 48, and 72 h (results represent a mean ± SD, $n = 3$)[AQ: 12]

more specific staining in kidney may give us definitive answers regarding the presence of FITC-labeled CMCht/PAMAM dendrimer nanoparticles in this organ.

One of the possible applications of the developed nanoparticles is to act as a nanocarrier of therapeutic molecules for targeting the CNS. In fact, delivery of drugs/vectors within the CNS is particularly challenging. In the brain, the presence of the FITC-labeled CMCht/PAMAM dendrimer nanoparticles was observed in the choroid plexus (CP) and intravascular space of small vessels within the parenchyma (Figure 1). Interestingly, there is an evidence that the nanoparticles possibly have passed to the ventricular space, as shown in Figure 1, suggesting that FITC-labeled CMCht/PAMAM dendrimer nanoparticles are able to cross the blood–CP–CSF barrier and access the ventricular space from the intravascular space. In order to confirm its presence, CSF was collected and further analyzed by flow cytometry (Figure 2). The results revealed the presence of FITC-labeled nanoparticles in CSF of animals within the 'high-dose' group. We observed 0.11% ± 0.03%, 0.16% ± 0.02%, and 0.27% ± 0.05% of FITC particles after 24, 48, and 72 h injection of dendrimer nanoparticles, respectively. Data are expressed as mean and standard deviation (SD). No dendrimer particles were observed in the CSF collected from the animals of the 'low-dose' group. These data corroborate histological sections findings, that is, they indicate that the concentration of 1 μg/g used for the low-dose group is not sufficient to obtain a wide *in vivo* biodistribution, including the CSF.

In spite of their presence in the ventricular space, neither neurons nor glial cells were able to internalize the FITC-labeled CMCht/PAMAM dendrimer nanoparticles, which can be explained by a low concentration of nanoparticles within the CSF and the presence of the CSF–brain barrier. Indeed, the physiologic barriers of the brain ▭ namely, the blood–brain barrier (BBB) are well known to impose a great difficulty in the application of new drugs to CNS.[17] Endocytosis and specific receptor-mediated transport mechanisms have been explored as potential routes for drugs to cross the BBB.[18] In order to overcome this issue, ligands and antibodies directed to specific receptors, present in BBB, such as transferrin,[19] may be linked to the terminal groups on the surface of CMCht/PAMAM dendrimer nanoparticles rendering them more efficacy and specificity to reach the CNS.

This principle may also be applied to other organs since the FITC-labeled CMCht/PAMAM dendrimer nanoparticles did not display any specificity to the organs/cells studied. The future applications
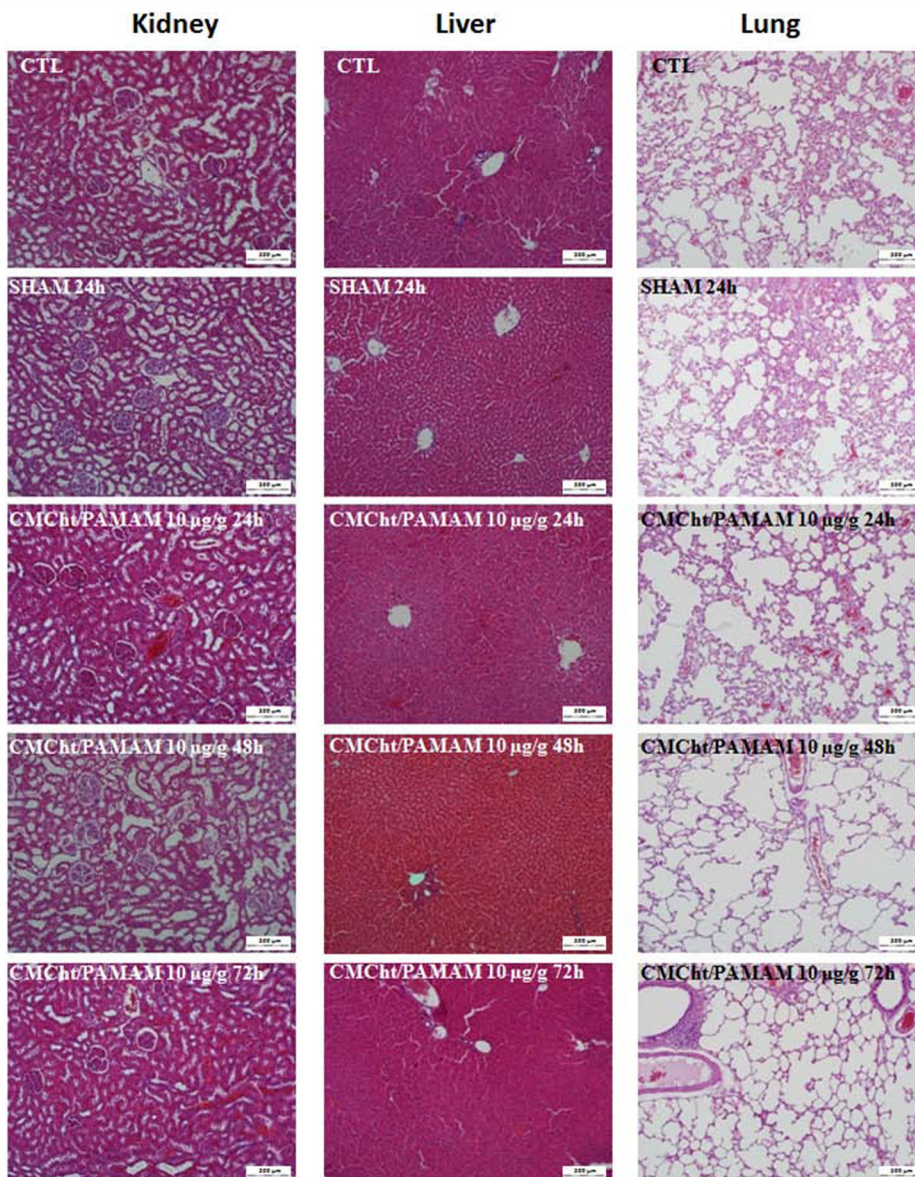
**Figure 3.** Histological appearance of the different organs studied for the three groups: sham, controls, and high-dose group (10 µg/g). The fluorescence micrographs show no major differences among the groups in terms of gross architecture, which is an indication of the lack of toxicity of the CMCht/PAMAM dendrimer nanoparticles

may be even broader since the nanoparticles may be also used as contrast carriers in bioimaging and gene transfection systems, among others.

Apart from understanding the location of the CMCht/PAMAM dendrimer nanoparticles within the different tissues/organs, it is also of the utmost importance to understand the toxicological consequences at the histological level. Figure 3 shows the results obtained for kidney, liver, and

lung. As it can be observed, no gross histopathological changes (namely, necrosis and/or inflammation) were detected among the experimental groups. These results suggest that the presence of the nanoparticles under study did not affect the structure of the different organs studied, disclosing, on this sense, a nontoxic profile for the observed organs.

## Conclusion

FITC-labeled CMCht/PAMAM dendrimer nanoparticles were injected in tail vein of young adult rats and traced in liver, lung, kidney, and brain. The results showed that these nanoparticles are stable in circulation for at least 72 h. After 24 h, their presence can be observed in the parenchyma; vasculature; and intracellular compartment of the liver, lung, and kidney. Moreover, there is evidence of their presence in the CP and ventricular space of the brain. It was also found that these results are concentration dependent as no positive results were obtained when a concentration of 1 µg/g of animal (instead of 10 µg/g) was used. The histological analyses showed no major disruptions of the architecture of these organs in the presence of the CMCht/PAMAM dendrimer nanoparticles. These data are an indication of their low-toxicity profile. In summary, this study provides for the first time information on the biodistribution and possible IV administration of the CMCht/PAMAM dendrimer nanoparticles *in vivo* and demonstrated their potential as multifunctionalized polymers for the design of intracellular drug delivery systems.

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