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Research Article

PEGylated Single-Walled Carbon Nanotubes as Nanocarriers for Cyclosporin A Delivery

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Abstract. Single-walled carbon nanotubes (SWCNTs) have attracted the attention of many researchers due to their remarkable physicochemical features and have been found to be a new family of nanovectors for the delivery of therapeutic molecules. The ability of these nanostructures to load large amounts of drug molecules on their outer surface has been considered as the main advantage by many investigators. Here, we report the development of a PEGylated SWCNT-mediated delivery system for cyclosporin A (CsA) as a potent immunosuppressive agent. The available OH group in the CsA structure was first linked to a bifunctional linker (i.e., succinic anhydride) in order to provide a COOH terminal group. CsA succinylation process was optimized by using the modified simplex method. The resulting compound, CsA–CO–$(CH_2)_2$–COOH, was then grafted onto the exterior surface of SWCNTs, previously PEGylated with phospholipid–PEG_{5000}–NH_2 conjugates, through the formation of an amide bond with the free amine group of PEGylated SWCNTs. Drug loading, stability of the PEGylated SWCNT–CsA complex, and *in vitro* release of the drug were evaluated. Loading efficiencies of almost 72% and 68% were achieved by UV spectrophotometry and elemental analysis methods, respectively. It was observed that 57.3% of cyclosporine was released from CsA–Pl–PEG_{5000}–SWCNTs after 3 days. In this investigation, we conjugated CsA to an amine-terminated phospholipid–polyethylene glycol chain attached on SWCNTs *via* a cleavable ester bond and demonstrated the possible potential of PEGylated SWCNT-based systems for CsA delivery.

KEY WORDS: carbon nanotubes; cyclosporin A; drug loading; elemental analysis; functionalization.

INTRODUCTION

Nanotechnology, as a very promising technology, has revolutionized the pharmaceutical and medical fields. The use of nanotechnology in the field of pharmaceutics and drug delivery has remarkably grown over the last few years, such that the pharmaceuticals developed on the basis of this technology are termed as *nanopharmaceuticals*. Nanoparticle drug-delivery systems, including polymeric nanoparticles, liposomes, dendrimers, nanoshells, carbon nanotubes, etc., offer the potential for improved efficacy of cancer diagnosis, treatment, and management. They also offer a promise for the targeted delivery of drugs, genes, and proteins to tumor tissues and therefore reduce the toxicity of anticancer agents in healthy tissues (1–3).

Carbon nanotubes (CNTs) were first recognized and introduced in 1952, and since their rediscovery in 1991, they have opened new horizons in the field of nanotechnology and nanomedicine (4). CNTs are synthetic nanomaterials which belong to the family of fullerenes and are made from rolled sheets of graphene built from sp^2-hybridized carbon atoms. These well-ordered, tubular structures with a diameter in nanometer range are classified as single-walled CNTs (SWCNTs) and multi-walled CNTs (MWCNTs). The former contain one layer of graphene sheet with a diameter of 0.5–2 nm, whereas the latter consist of multiple layers of graphene rolled simultaneously to form concentric tubes. The CNT lengths vary greatly depending upon the synthesis conditions and can reach values as high as several centimeters, thus having extremely high aspect (length-to-diameter) ratios (5,6).

Due to unique architecture and outstanding physicochemical properties, CNTs have been the subject of a large number of researches and projects describing their applications in the fields of biology, pharmaceutics, and medicine. These features have made them promising tools to revolutionize human disease diagnosis and treatment (7–32). Among the several fascinating properties that may be exploited in biomedical applications, one can mention ultrahigh surface area and high capacity for drug loading through integration of targeting molecules onto the surface by either covalent or non-covalent interactions (5,33); easy internalization by cells, thereby providing the opportunity to target drugs to tumor sites (5); optical properties such as near-infrared florescence

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and absorption (leading to localized hyperthermia and the death of tumor cells), Raman scattering and photoacoustic properties (useful for bio-imaging and disease diagnosis) (5,34,35).

Naturally, CNTs are hydrophobic and thereby show low dispersibility in almost all organic solvents as well as aqueous media. This makes their application in biological and life sciences rather difficult (1,5,18,20,25). This chemical characteristic is thought to be attributed to the smooth surface with no hanging bonds, rather hydrophobic character of the sidewalls, coupling the individual tubes, and the formation of aggregates. However, several strategies have been developed to modify the CNTs' graphitic sidewalls, enabling the attachment of various chemical and biochemical moieties on the nanotubes' surface. The two main approaches are based on either covalent or non-covalent functionalization of CNTs with surfactants, block copolymers, and biopolymers (4,6,7,9,33,34,36). In addition to rendering hydrophilic character to CNTs so as to avoid their aggregation and increase the dispersibility in aqueous solution and biocompatibility, functionalized SWCNTs and MWCNTs have been found to bypass effectively the biological barriers with a high cellular uptake. This would enable them to play a role of cargo in delivery of therapeutically active molecules into the cells (34,37,38). On the other hand, functionalization of SWCNTs could optimize the blood circulation time, selectivity, and retention in the tumor, and reduces the intrinsic toxicity and immugenicity which are required properties for drug delivery (5,25,34,39–45).

The distinct structural properties of carbon nanoparticles, in particular their high aspect ratio, propensity to functional modification, their potential biocompatibility and nanofluid nature, make them useful for nano- and controlled drug delivery. The possibility of simultaneous grafting of several pharmaceutically active ingredients onto the exterior surface of functionalized CNTs, absorption and entrapment of the active component within CNT bundles, or encapsulation of molecules inside the nanochannel of CNTs, and therefore creating CNT conjugates endowed with pharmacological activities, have attracted much attention. In recent years, various types of drug delivery or controlled release systems based on CNTs have been developed. Small drug molecules were attached to the CNT surface by covalent conjugation to the functional groups or to the polymer coating of CNT groups (25). This conjugation was mostly designed to be *via* cleavable bonds (37,46–52). As an alternative strategy, aromatic molecules with flat structures could be adsorbed on the surface of CNTs through non-covalent π–π stacking (46,53,54). These two approaches have offered enhanced tumor cell permeability, increased drug accumulation in the tumor, and, in turn, improved treatment efficacy and outcome of anticancer drugs loaded on CNTs. Intracellular delivery of biomacromolecules such as proteins, DNA, and RNA through side wall conjugation or non-covalent adsorption onto the hydrophobic surface of non-functionalized CNTs has also been investigated (34,55,56).

The pharmacokinetics and biodistribution of CNTs have been studied since the safety issue of CNTs was proposed. Singh *et al.* and Lacerda *et al.* have determined the biodistribution of radiolabeled (111In-DTPA)-SWCNTs functionalized by 1,3-dipolar cycloaddition following the intravenous injection. They observed a fast urinal clearance of SWCNTs (>95%) within 3 h with no uptake in RES organs (57,58). Pharmacokinetics and biodistribution of CNTs can be improved significantly by carefully choosing suspending reagents. It has been found that biocompatible PEGylated SWCNTs are mostly accumulated in the liver and spleen after intravenous administration, with slow excretion probably *via* the biliary pathway into the feces. It has also been reported that long polyethylene glycol (PEG) coatings would generally prolong the blood circulation half-life, reduce the uptake in RES organs, and accelerate the excretion of SWCNTs. Branched PEGs may offer a longer blood circulation half-life through more efficient surface coating, compared to linear PEGs with the same molecular weight (31,59). Liu and his colleagues reported that PEGylation of SWCNTs with phospholipid (Pl)–PEG_{2000} could increase the half-life to 1.2 h, whereas the circulation half-life of SWCNTs could be increased to about 5 and 15 h when Pl–PEG_{5000} and branched PEGs were used as suspending agents, respectively (60).

The aim of the main project was to use SWCNT-based nanostructures as efficient carriers for loading and delivery of cyclosporin A (CsA). To overcome the problems associated with the solubility of SWCNTs, the first step of the research was planned to functionalize SWCNTs non-covalently with two commercially available amine-terminated phospholipid polyethylene glycols (namely Pl–PEG 2000 and Pl–PEG_{5000}) (61). Evaluation of functionalized SWCNTs showed that our non-covalent functionalization protocol could considerably increase aqueous solubility. As the second step of this study, we hypothesized that through non-covalent functionalization with Pl–PEGs, the toxicity of the Pl–PEG–SWCNT conjugates would be considerably reduced, and therefore, an investigation was planned to study the effect of SWCNT PEGylation on viability and proliferation of the Jurkat cell line. We found that PEGylated SWCNTs were substantially less toxic, compared to pure SWCNTs (62). Based on the results obtained previously, in the third part of the investigation, we decided to use Pl–PEG_{5000}–SWCNTs as nanovectors for the delivery of CsA. In this regard, the OH group in the CsA structure was esterified by using succinic anhydride as a linker through a chemical reaction, and the yield of this reaction was then optimized using the modified simplex technique. The resulting compound (CsA–CO–$(CH_2)_2$–COOH) was then grafted onto the exterior surface of Pl–PEG–SWCNTs through the formation of an amide bond with the free amine group of PEG molecules. Finally, drug loading and stability of the CsA–Pl–PEG_{5000}–SWCNT complex and *in vitro* release of drug were then evaluated.

MATERIALS AND METHODS

Materials

SWCNTs (P2-SWCNTs) and Pl–PEG_{5000}–NH_2 were purchased from Carbon Solutions Company (USA) and NOF-Sunbright Company (USA), respectively. CsA was obtained from Zahravi Pharmaceutical Company (Iran). Dry pyridine, 4-dimethylaminopyridine, and succinic anhydride (purity ≥ 99%) were purchased from Sigma-Aldrich (Germany), and 1-ethyl-3 [3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), *N*-hydroxysulfosuccinimide (sulfo-NHS), 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer saline, and

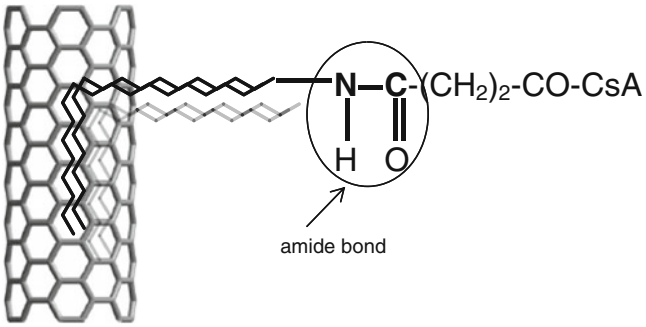

Fig. 1. Schematic presentation of indirect conjugation of a CsA molecule onto the exterior surface of functionalized SWCNTs (with Pl–PEGs) *via* succinic anhydride as a linker

phosphate buffer saline (PBS) were all obtained from Thermo Scientific Company (USA).

Succinylation of OH Functional Group in CsA

In this study, CsA was covalently attached to Pl–PEG–SWCNTs *via* a succinyl spacer group. Succinic anhydride, a bifunctional spacer, was used to introduce a carboxyl acid group on the CsA molecule at the C′-2 OH position. CsA was added to succinic anhydride with the molar ratios of 2–6 in the presence of dry 4-dimethyaminopyridine. An appropriate amount of anhydrous pyridine was then added, and the mixture was stirred for 3–6 h under the atmosphere of nitrogen gas at 25 or 50°C. The formation of succinyl-CsA derivative (CsA–CO–$(CH_2)_2$–COOH) was confirmed by liquid chromatography–mass spectroscopy (LC-MS; LC 1200 Agilant, MS 6410 Triple Quad Agilant, equipped with Zobrax 10×2.5 cm, 5 μm column, USA) on the basis of detecting weight difference between CsA and succinyl-CsA derivative.

Process Optimization Using Modified Simplex Method

The CsA succinylation process was optimized by using the modified simplex method. Four independent variables including succinic anhydride/CsA molar ratio (X_1), the amount of 4-dimethylaminopyridine (X_2), the stirring time (X_3), and the temperature (X_4) were selected for running the reactions according to the modified simplex algorithm. The area under the curve of the succinyl-CsA peak, which appeared in m/z of 1,324 in the LC-MS chromatogram, was defined as the dependent variable (response) for further approaching to optimum reaction conditions to achieve a high yield of 2′-succinyl-CsA production.

Conjugation of Succinyl-CsA with Pl–PEG_{5000}–SWCNTs

Five millimolars of EDC and 5 mM of sulfo-NHS were added to 300 nM of 2′-succinyl-CsA in MES buffer saline (pH=6), and the solution was stirred for 30 min at room temperature. In the next step, 1 mL of 0.3 mM Pl–PEG_{5000}–SWCNTs PBS solution (pH=7.4) was then added to the solution while stirring. After 6 h, the resulting mixture was ultrafiltered using Vivaspine 2 centrifugal filter device with a molecular weight cutoff of 5 kDa in order to remove CsA–Pl–PEG_{5000}–SWCNT conjugates from unconjugated free CsA.

UV–Vis Spectrophotometry

A complete wavelength scan over the UV region was performed. The UV spectra of free succinyl-CsA derivative and CsA–Pl–PEG–SWCNTs within the range of 200–400 nm showed that there was a 20-nm shift in λ_{max}, and therefore, the wavelength of 260 nm was selected for the evaluation of the drug loading on SWCNTs. An ultrafilter device with a molecular weight cutoff of 5 kDa was used to separate the free succinyl-CsA derivative from CsA–Pl–PEG–SWCNTs. Various concentrations of CsA–Pl–PEG_{5000}–SWCNTs in PBS were prepared and filtered. The clear filtered samples were used for the construction of a calibration curve, using a UV–vis spectrophotometer (CECIL CE 2021, 2000 series, England), at the wavelength of 260 nm. By linear regression analysis of the corresponding plot, a linear curve was obtained and used for the calculation of CsA loaded onto SWCNTs. The same technique was used for the construction of a standard calibration curve for CsA at 280 nm.

Elemental Analysis

Elemental analysis (ECS 4010 CHNOS Analysis, Costech International S.p.A, Italy) was applied to confirm the CsA loading on PEGylated SWCNTs. This method can declare, qualitatively and quantitatively, the changes that occur in both type and percentage of present elements in pure SWCNTs, CsA, Pl–PEG_{5000}, Pl–PEG_{5000}–SWCNTs, and CsA–Pl–PEG_{5000}–SWCNTs.

Drug Release from CsA–Pl–PEG_{5000}–SWCNT Conjugates

Suspensions of CsA-loaded SWCNTs in acetate buffer saline (pH=5.5) with various concentrations (0.06–1 mg/mL) were prepared and filtered. The clear filtered samples were used for the construction of a calibration curve. Samples with known concentrations were allowed to stand at 37°C for up to 72 h. At predetermined intervals (i.e., 24, 48, and 72 h), the

Table I. Various Conditions Selected for the Synthesis of Succinyl-CsA

No.	Succinic anhydride–CsA molar ratio (mg)	Dimethylaminopyridine (mg)	Stirring time (h)	Temperature (°C)	Dry pyridine (mL)
1	0.2:0.1 mM (20.14:120.3)	0.01 mM (1.2)	0.2	50	3
2	0.2:0.066 mM (20.14:79.4)	0.025 mM (3.1)	0.2	25	4
3	0.2:0.05 mM (20.14:60.13)	0.055 mM (6.7)	0.3	50	5
4	0.2:0.04 mM (20.14:48.1)	0.055 mM (6.7)	0.4	25	6
5	0.2:0.033 mM (20.14:39.7)	0.04 mM (4.9)	0.4	25	3
6	0.2:0.05 mM (20.14:60.13)	0.04 mM (4.9)	0.3	50	6

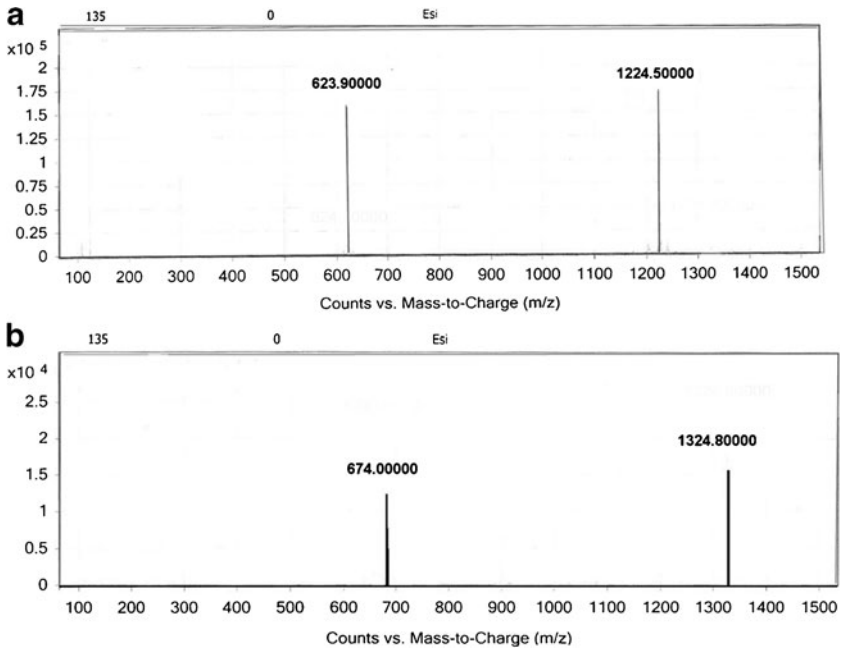

Fig. 2. LC-MS chromatograms of **a** CsA and **b** succinylated CsA

mixture was ultrafiltered using Vivaspine 2 centrifugal filter device with a molecular weight cutoff of 5 kDa. The concentration of the released CsA in the filtrate and the remaining amount of CsA–Pl–PEG_{5000}–SWCNTs were determined by UV–vis spectrophotometry (CECIL CE 2021, 2000 series, England).

RESULTS

CsA is a lipophilic, cyclic polypeptide and highly rich in amide bonds. In its chemical structure, there is only one hydroxyl functional group that could be utilized for the attachment process. Based on this chemical structure, various strategies were first theoretically studied for the attachment of CsA onto the surface of SWCNTs, among which the indirect grafting of the drug *via* its hydroxyl group by using amine-terminated Pl–PEGs was selected as the main strategy and investigated experimentally. It was assumed that the esterification of the OH group in CsA by a bi-functional linker such as succinic anhydride and therefore formation of a COOH group could provide the possibility to make a second conjugation through the formation of an amide bond between the free amine group of Pl–PEG–SWCNTs and COOH. It was also assumed that the cleavable esteric bond would facilitate the release of CsA from the Pl–PEG–SWCNT backbone. Figure 1 illustrates schematically the planned strategy for the indirect attachment of CsA on the external surface of functionalized SWCNTs *via* the linker investigated.

Preliminary reactions were run in order to investigate the important factors that affect the succinylation process (Table I). LC-MS was employed to verify the synthesis of succinyl derivative. As it is shown in Fig. 2, CsA is characterized with mono sodium adduct and double sodium adduct peaks that appeared in m/z of 1,224 and 623, respectively, whereas the succinyl-CsA derivative is characterized with mono sodium adduct and double sodium adduct peaks observed in m/z of 1,324 and 674, respectively. The mass difference indicates the formation of a covalent bond between CsA and succinic acid.

In the next step, it was planned to optimize the yield of the succinylation process with the help of the modified simplex algorithm. Four factors, including succinic anhydride/CsA molar ratio (X_1), the amount of dimethylaminopyridine (X_2), stirring time (X_3), and the temperature (X_4), were selected to be optimized at constant concentration of anhydrous pyridine. The area under the curve of the mono sodium adduct peak which appeared in m/z of 1,324 was defined as the response to be optimized, and our strategy was to maximize

Table II. Designed Reactions for Succinyl-CsA Formation on the Basis of Modified Simplex Algorithm

No.	Succinic anhydride–CsA molar ratio (mg) (X_1)	Dimethylamino pyridine (mg) (X_2)	Stirring time (h) (X_3)	Temperature (°C) (X_4)	AUC (1,324.8)
1	0.2:0.1 mM (20.1:120.0)	13,204,990	40	3	0.01 mM (1.2)
2	0.3:0.1 mM (30.2:120.0)	17,416,732	25	6	0.025 mM (3.1)
3	0.4:0.1 mM (40.2:120.0)	25,365,830	40	6	0.055 mM (6.7)
4	0.5:0.1 mM (50.2:120.0)	14,255,197	25	3	0.055 mM (6.7)
5	0.6:0.1 mM (60.2:120.0)	21,197,713	25	6	0.04 mM (4.9)
6	0.25:0.1 mM (25.1:120.0)	15,268,040	40	3	0.04 mM (4.9)

CsA cyclosporin A, *AUC* area under the curve

Table III. Results of Elemental Analysis Obtained for Pure SWCNTs, Pl–PEG 5000–NH2, CsA, Pl–PEG 5000–SWCNTs, CsA–Pl–PEG 5000, and CsA–Pl–PEG 5000–SWCNTs

Element	SWCNTs	Pl–PEG_{5000}–NH_2	Pl–PEG_{5000}–SWCNTs	CsA	CsA–Pl–PEG_{5000}–SWCNTs	CsA–Pl–PEG_{5000}
N	–	0.7%	2.82%	10.11%	12.5%	8.2%
C	85%	55.37%	67.68%	55.67%	69.1%	58.5%
H	–	8.72%	3.57%	7.49%	9.5%	9.2%

SWCNT single-walled carbon nanotube, *Pl* phospholipid, *PEG* polyethylene glycol

this AUC which was indirectly relevant to 2-succinyl ester production, based on the designed conditions for the reaction. Results showed that the succinic anhydride/CsA molar ratio of 3–6, 0.055 mM of dimethylaminopyridine, and 6 h of stirring at 30–40°C were the optimized conditions for the reaction. Table II summarizes the designed reactions for CsA–CO–$(CH_2)_2$–COOH formation on the basis of the modified simplex algorithm.

Amidation reaction was then performed in the presence of EDC as a highly reactive carbodiimide cross-linker at a given pH, using sulfo-NHS as the stabilizing agent to improve the coupling process and increase the conjugation yield. UV–vis spectrophotometry revealed that Pl–PEG–SWCNTs, CsA, and CsA–Pl–PEG_{5000}–SWCNTs have maximum wavelengths at 245, 280, and 260 nm, respectively. Therefore, loading efficiency was evaluated by this technique and estimated to be 71.89% for Pl–PEG_{5000}–SWCNTs.

Elemental analysis was employed to evaluate, qualitatively and quantitatively, the CsA coupling to Pl–PEG_{5000}–SWCNTs. Pure SWCNTs consist of only carbon atoms. However, covalent or non-covalent functionalization would change the elemental content to a great extent. In most situations, carbon content would decrease dramatically. In the present study, non-covalent functionalization of SWCNTs with Pl–PEGs and CsA loading would lead to a decrease in carbon content and an increase in other elements such as nitrogen and hydrogen. Elemental contents of pure SWCNTs, Pl–PEG_{5000}–NH_2, Pl–PEG_{5000}–SWCNTs, CsA, CsA–Pl–PEG_{5000}, and CsA–Pl–PEG_{5000}–SWCNTs are shown in Table III. As depicted, carbon percentage for pure SWCNTs and CsA–Pl–PEG were estimated to be 85% and 61.5%, respectively. CsA loading on Pl–PEG_{5000}–SWCNTs was then calculated by the following equation, using an iteration method:

$$\text{Percent of carbon in CsA}-\text{Pl}-\text{PEG}_{5000}-\text{SWCNT complex} = (0.85 \times \text{A}) + (0.615 \times \text{B})$$

where *A* and *B* are the contributions of SWCNTs and CsA–PEG in the complex, respectively. Calculations showed that almost 68% of CsA–Pl–PEG were loaded on SWCNTs.

Since the functionalized SWCNTs mainly entered the cells through the process of endocytosis (31,42,50,54,63–69), the pH of the medium for the release of the drug was adjusted at 5.5 (the pH of lysosomes), using acetate buffer saline. As mentioned earlier, samples with known concentrations were allowed to stand at 37°C for up to 72 h. It was observed that CsA was released slowly from the CsA–Pl–PEG_{5000}–SWCNT complex for 48 h, whereas a faster trend occurred within 48–72 h (Fig. 3).

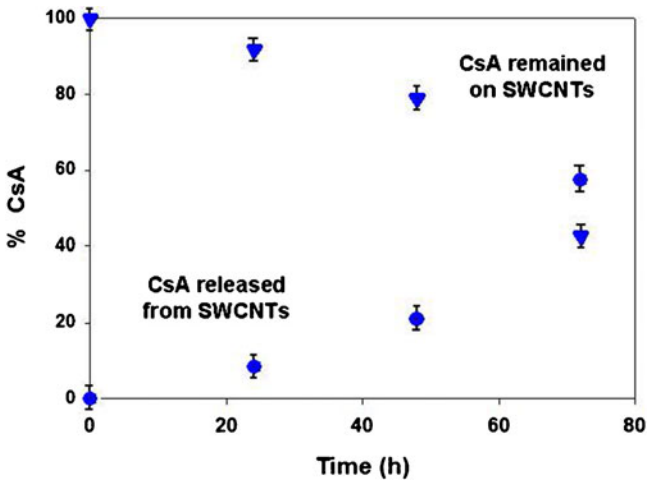

Fig. 3. The amount of CsA released from the CsA–Pl–PEG_{5000}–SWCNTs complex and the CsA residual on the complex *versus* time after 24, 48, and 72 h at 37°C in acetate buffer saline (n=3)

DISCUSSION

Among numerous nanopharmaceuticals introduced in the drug delivery platform, only some of them have gained widespread applications. SWCNTs, either single walled or multiple walled, are a new, emerging class of materials with several important properties and could be considered as promising nanovectors for targeting drug delivery, treating cancer, and other diseases. However, lack of solubility in organic solvents and aqueous media, bundle formation, short circulation half-life, biocompatibility issues, and immunogenicity limitations have indicated the need for further modifications of these novel nanocarriers. In this regard, feasibility of functionalized CNTs (*f*-CNTs) as safe bio-nano-materials has been proposed by various research groups. The two main approaches are based on either non-covalent or covalent functionalization of carbon nanotubes with surfactants, polymers, and biologic molecules.

Owing to its biocompatibility, inert properties, water solubility, and good stability under high salt and protein concentration, PEG derivatives have been used for various purposes in biomedical applications. PEGylated CNTs are supposed to have longer blood circulating time, reduced reticuloendothelial system uptake, and less nonspecific binding of serum proteins (9,31). Non-covalent functionalization of SWCNTs by Pl–PEG_{5000} conjugates was first developed by our group, and *in vitro* cytotoxicity studies were then performed in order to investigate whether the functionalization with Pl–PEGs could have any effect on SWCNT toxicity on cultured Jurkat cells (62).

CsA is prescribed intravenously or orally for the prophylaxis of organ rejection in kidney, liver, and heart allogeneic

transplants. Poor aqueous solubility, narrow therapeutic window, high volume of distribution, high plasma protein binding, low bioavailability, and high inter- and intra-individual variability have made it necessary to investigate other potential delivery systems. However, an ideal delivery system for CsA exhibiting high loading capacity and bioavailability, and targeted delivery to the site of the allograft following systemic administration with sustained release rate has yet to be designed (70,71).

The present study was designed to examine whether or not Pl–PEG–SWCNTs would be potentially suitable as nanocarrier for CsA delivery. Pl–PEGs are amphiphilic compounds with both hydrophobic side chains and hydrophilic charged head group. The hydrophilic domain extending into the aqueous phase not only causes desirable water solubility, but also provides functional groups for drug conjugation. It should be noted that Pl–PEGs, with one available amine group applied in our study, would reduce the possible steric hindrance, compared to multi-armed Pl–PEGs. This would be of great importance for loading of molecules with three-dimensional structures and special configurations with hidden chemically reactive groups (e.g., CsA). Considering the chemical structure of CsA, the indirect attachment of the free hydroxyl group to the free amine group of Pl–PEG_{5000} seemed to be the effective strategy. To do this, succinic anhydride was used as a linker to react with the OH group of CsA. In this reaction, CsA was first succinylated and converted to a monoester with a free COOH group which was assumed to interact with the amine group of Pl–PEGs in the presence of catalyzers (i.e., EDC and sulfo-NHS), leading to the formation of CsA–Pl–PEG_{5000}–SWCNTs through the formation of an amide bond. EDC is a carbodiimide cross-linker and highly reactive with OH and amine groups. EDC reacts with the COOH group of succinylated CsA and forms *O*-acyl urea as an intermediate compound which is unstable in aqueous medium. In order to improve the coupling process and achieve the appropriate yield of conjugation, amidation reaction was performed in the presence of sulfo-NHS as the stabilizing agent. This strategy was selected in this research because it was hypothesized that the cleavable esteric bond would facilitate the release of CsA from the Pl–PEG–SWCNT backbone. It should be noted that the stability of the CsA–Pl–PEG_{5000}–SWCNT complex in phosphate buffer saline (pH=7.4) was evaluated after 4 weeks of storage at 2–8°C, and the amount of CsA released from the complex was regarded as instability evidence. We observed that the rate of hydrolysis of the ester bond to release CsA was significantly slower at pH=7.4 than that at pH=5.5, which is the appropriate pH for the release of the drug, once the complex has entered the cells through the process of endocytosis.

CONCLUSION

Great attempts have been made in the development of SWCNTs as advanced nanomaterials for biomedical applications. Studies performed in our laboratories and other investigations have indicated that SWCNTs could be considered as promising nanovectors for the delivery of a variety of therapeutic agents, following their surface modification to increase their water dispersibility, render them biologically compatible, decrease their toxicity, and increase their ability to cross the cell membranes. We first planned to PEGylate SWCNTs with Pl–PEG_{5000} through hydrophobic interaction between CNT sidewalls and the hydrocarbon chain of the phospholipid component of a Pl–PEG moiety in order to obtain non-covalent functionalized Pl–PEG_{5000}–SWCNTs. Then, following the cytotoxicity studies, our observations confirmed that the non-covalent PEGylation protocol proposed in this project could improve the SWCNTs' biocompatibility significantly. The present study was planned to use PEGylated SWCNTs for the delivery of CsA. Although lacking in certain aspects, the results obtained by our research until now clearly showed the potential of PEGylated SWCNT-based systems for drug delivery. Future studies will determine the limitations and opportunities of this CsA-loaded PEGylated SWCNTs for clinical applications.

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Conflict of Interest The authors report no conflicts of interest in this work.

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