

Multifunctional Curcumin-Nanocarriers Based on Host-Guest Interactions for Alzheimer Disease Diagnostic

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Abstract

The accumulation of amyloid peptides 40 and 42 in senile plaques is one of the hallmark lesions of Alzheimer Disease (AD). Great efforts are currently made to design molecules able to target these lesions in brain, both for diagnostic and therapeutic aims. Recent studies showed that curcumin has high affinity for the amyloid deposits. Curcumin is a fluorescent molecule with wide pharmaceutical activities, including potent anti-oxidant, anti-inflammatory, and anti-carcinogenic properties. Still, its low solubility limits its clinical and preclinical use. The use of controlled stoichiometric ratios between curcumin and host molecules like Methyl- β -cyclodextrin, *para*-sulphonato-calix(4)arene and *para*-sulphonato-calix(6)arene allowed the solubilisation of curcumin and the formation of stable nanocarriers. Within the nanocarriers, curcumin was available at their surfaces, being able to interact with the environment. They showed high affinity for the amyloid deposits, strongly labeling not only the senile plaques but also the diffuse deposits of Alzheimer Disease brains. Their biocompatibility was proved on several cell lines. Moreover, they were shown to interact with the A β peptide, reducing its aggregation and preventing its toxicity.

Keywords: Alzheimer disease; Amyloid peptide aggregation; Curcumin; Macromolecules; Nanocarriers

Introduction

Alzheimer Disease (AD) is one of the most common forms of dementia, currently affecting more than 26 million people worldwide. The two pathological features characterizing AD are the intraneuronal accumulation of tau protein (neurofibrillary tangles) and the extracellular aggregation of the Amyloid beta peptide, (A β), (senile plaques). Their detection in *post-mortem* tissue is still indispensable for the diagnosis [1]. The A β peptide, the main component of senile plaques, is considered to be the driving actor in the progression of Alzheimer Disease. According to the amyloid cascade hypothesis [2,3], the accumulation of A β peptide in the brain is the initial event in the succession of reactions that lead to cortical dysfunction. A β plaques are present in moderate to frequent numbers in the cortical grey matter of AD patients, years before the onset of dementia. The possibility of efficiently target A β pathology in the brain is an important strategy for developing solutions for early diagnosis. Still, the development of biocompatible probes with selective affinity for the amyloid plaques is a challenge.

Several studies showed the potential of curcumin in the treatment of AD. Curcumin not only impedes the aggregation of the amyloid peptide but is also able to disaggregate the already formed fibrils, according to *in vitro* studies [4-7]. Curcumin, as well as curcumin derivatives, already proved their potential for the diagnosis of AD, being able to bind to the amyloid deposits *in vitro*, *in vivo* or on *post-mortem* tissue [8-13]. Moreover, curcumin is known to have numerous other protective and curative properties: anti-oxidant [14-19], anti-inflammatory [20-22], and anti-cancerous [23-27], confirming its potential bio-compatibility.

Despite such encouraging reports, the study of curcuminoids is severely limited by their exceedingly low bioavailability, due to their poor solubility and instability in aqueous solutions. Thus, curcuminoids are often prepared in dimethyl sulfoxide (DMSO) or methanol, and

this has raised questions about their clinical efficacy. As the need for suitable probes for targeting A β aggregates in brain at early stages of AD is becoming imperative, efforts have been made to improve the solubility of these compounds [28].

Nanoparticle-based delivery has the potential of rendering hydrophobic agents, such as curcumin, dispersible in aqueous media. Liposomes and polymeric nanoparticles have been widely used as delivery systems [29-33]. An alternative to increasing the water solubility of active principles is the complexation with macromolecules. Cyclodextrins [34] and calix(n)arenes [35] are the most studied classes of macrocyclic host-compounds in supramolecular chemistry [36]. The cyclodextrins (α -, β -, γ -cyclodextrins) and their derivatives (2-O-methyl β -CD hydroxypropyl- β -CD and hydroxypropyl- γ -CD) were already proved to complex curcumin [37-43], improving its solubility by about 100 times. *Para*-sulphonato-calix(4)arene was also recently shown to be able to stabilize curcumin in aqueous solutions. The improved bio-activity of curcumin-cyclodextrin complexes was confirmed by several studies [37-40,42]. Two recent studies reported on the therapeutic effect of curcumin-cyclodextrin formulation *in vivo*, in AD mice models [43,44]. Still, up to now, the possibility to solubilize curcumin with the aid of macromolecules without impeding its ability

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to target the amyloid peptide and the amyloid deposits, for potential application in the early diagnosis of AD has not been evaluated.

Here we report a new stealth and efficient solubilisation of curcumin using the *para*-sulphonato-calix(4)arene (SC4), the *para*-sulphonato-calix(6)arene (SC6) and Methyl β -cyclodextrin. These nanoparticles were able to strongly label various amyloid aggregates in AD brains proving their potential as trackers of AD pathology.

Experimental

Materials

Curcumin and Methyl β -cyclodextrin (M β CD) were obtained from SIGMA and were used as received. The *para*-sulphonato-calix(4)arene (SC4) and the *para*-sulphonato-calix(6)arene (SC6) were synthesized according to the method of Coleman et al, by direct sulphonation of calix(n)arene [45].

Curcumin solubilisation

Curcumin was solubilized in ethanol at a concentration of 3 mM. A volume of 5 ml of macromolecules in water (at 300 μ M concentration) was added, drop by drop, to 500 μ l of curcumin solution, under continuous stirring. Thus, a final molar ratio of 1:1 between curcumin and the macromolecules was obtained. The formation of a colloidal phase is instantaneous. Stirring was allowed for 2 h, without a cap, to completely evaporate ethanol. The so obtained complexes: M β CD-curcumin (M β CD-Curc), SC4-curcumin (SC4-Curc) and SC6-curcumin (SC6-Curc) were kept at 4°C for 12 h, on a rotating wheel. The eventual curcumin precipitate was eliminated by centrifugation at 10000 rpm for 15 min. The loaded quantity of curcumin within the inclusion complexes was further estimated spectrofluorimetrically in 50% EtOH and was found to be of 20 μ g/ml for all the complexes. The colloids were further stocked at 4°C until use.

Molecular docking studies

The 3D molecular structures of curcumin and of the three inclusion complexes were obtained from the Cambridge database. The docking was performed with AutoDock Vina software and analyzed by PYMOL of the Scripps Research Institute. The receptor (M β CD, SC4 or SC6) and the ligand (curcumin) were built up independently and the inclusion complex model was determined by energy minimization.

Characterization of the complexes

The size of the supramolecular structures was estimated by dynamic light scattering, using a Malvern Nanosizer (particle sizer and ζ -potential analyser), with a 625 nm laser beam. The intensity autocorrelation functions of the light scattered at a fixed angle of 173° gave the size and the polydispersity indices. Transmission electron microscopy was employed to analyse the morphology of the complexes. Volumes of 1 μ l of each colloid were deposited on formvar-coated copper TEM grids. The morphology of samples was observed under JEOL-1210 transmission electron microscope (JEOL, Tokyo, Japan) operating at 60 kV.

The monodispersity of the supramolecular structures and the stability in time were evaluated by means of flow cytometry, using a Gallios® cytometer (Beckman Coulter), at room temperature.

Alzheimer disease cases

AD patients enrolled in a brain donation program of the Brain Bank "GIE NeuroCEB" run by a consortium of Patients Associations

(including France Alzheimer Association) were employed for this study. The consent for the research utilization was signed by the patient himself, or the next of kin, in accordance with the French Bioethical Laws. The corpse was transported to the mortuary of a University Hospital belonging to the Neuro CEB network at the time of death. The brain was removed; one hemisphere was fixed in buffered 4% formaldehyde for the neuropathological diagnosis of AD; the other was immediately sliced. Samples from the superior temporal gyrus (Brodmann area 22) were mounted on a cork piece, dipped in isopentane cooled by liquid nitrogen and kept in a deep freezer at -80°C until use. The present study has been approved by the *ad hoc* committee of the Brain Bank.

Diagnosis

Formalin-fixed 5 μ m thick sections, from multiple areas of the brain, including hippocampus and isocortical Brodmann area 22, were used for the diagnosis. The diagnosis of AD was confirmed by immunostaining with anti-A β antibody (6F3D clone; Dako, Trappes France) and anti-tau antibody (polyclonal rabbit anti-tau; Dako; Trappes code number A 0024). The diagnosis criteria of the NIA-Reagan Institute were used [46].

Affinity of curcumin complexes for the amyloid deposits

Sections from the temporal isocortex (superior temporal gyrus) of three AD subjects (Braak neurofibrillary stage VI, Thal phase 5), containing numerous amyloid deposits and neurofibrillary tangles were used for the study. *Post-mortem* frozen 10 μ m thick sections were prepared with the aid of a Leica cryostat. The samples were hydrated for 5 min with Phosphate Buffer Saline (PBS) and then incubated 2 h with 200 μ l of the curcumin-macromolecules solution at 1 μ g/ml. Subsequently, the samples were gently washed in PBS and mounted with a medium minimizing fading of the fluorescence (Dako Fluorescence Mounting Medium). In order to confirm the positive staining of amyloid deposits by curcumin-macromolecules complexes, A β immunohistochemistry was also performed before incubation with curcumin. After acetone fixation and PBS washing, the sections were incubated for 4 h in 200 μ l solution of 6F/3D antibody (Dako), at 1/200 dilution. The samples were washed 3 times with PBS and incubated for other 2 h with the secondary antibody bearing the chromogen red Cy3. Following the immunostaining, the samples were further washed in PBS before incubation with curcumin-macromolecule complexes, as previously described. The colocalization was examined using 488 nm (to detect the staining of curcumin) and 543 nm (for Cy3 detection) excitation wavelengths, the signals being collected between 540-550 nm and 565-580 nm, respectively. Colocalization was indicated by a yellow color on the "merged" images.

Cytotoxicity assay

To evaluate the cytotoxicity of curcumin complexes, control SH-SY5Y cells (human neuroblastoma cells), HEK cells (from Human Embryonic Kidney), hAPP_{sw} SH-SY5Y and hAPP_{sw} HEK cells - cells stably overexpressing the human APP gene (hAPP) bearing the Swedish mutation causing familial Alzheimer disease - were used. The cells were seeded in 96 multiwell culture plates and grown in DMEM-F12 media containing 10% of Fetal Calf Serum (FCS) until approximately 80% confluence. The growth medium was then discarded and the cells were incubated for 24 h in a culture medium enriched in curcumin complexes (final concentration of curcumin 200 ng/ml). The cytotoxicity was assessed by means of MTT test, based on the conversion of tetrazolium salt into a purple formazan product [47].

Rescuing effect

Control SH-SY5Y cells were used for determining the capacity of curcumin-macromolecule complexes to rescue the toxicity of A β 42. Cells were seeded in 96 multiwell culture plates and grown until approximately 80% confluence. At this point, the growth medium was replaced by a new medium to which a freshly prepared solution of A β 42 (purchased from GenicBio) in PBS (100 ng/ml) was added. The culture medium was also supplemented or not with curcumin-macromolecule complexes, with a final concentration of curcumin of 200 ng/ml. The cell viability was assessed by an MTT test, the experiments being carried six times in triplicates.

Aggregation assay

A solution of 5 μ M A β 42 in water was freshly prepared and allowed to spontaneously aggregate at 20°C. A volume/volume solution of curcumin-macromolecule complexes (5 μ g/ml final concentration in curcumin), or PBS (used as control) was added to the A β 42 solution. The effect of curcumin on the aggregation was measured by Thioflavin T assay [48], monitoring the fluorescence between 410-470 nm excitation and 475-495 nm emission wavelengths.

Results

Structural characterization of curcumin-macromolecule complexes

The M β CD macromolecule is a 7 membered α ,1-4glucose cyclic molecule, while SC4 and SC6 are macrocycles composed of respectively 4 and 6 phenolic rings, functionalized at the upper rim by sulphonate groups, favoring numerous electrostatic interactions, besides the hydrophobic aromatic interactions. The structural characteristics of curcumin and of its host macromolecules, as well as the model inclusion complexes are presented in Figure 1. In the complex with M β CD, one of the phenolic rings of the curcumin is engaged in the macrocycle, stabilized by hydrogen bonds with the hydroxyl groups. According to the molecular docking, SC4 is able to complex 2 curcumin molecules. One of the molecules, cinctured the macrocycle, two phenolic rings being involved in π - π interactions with the host. Another curcumin

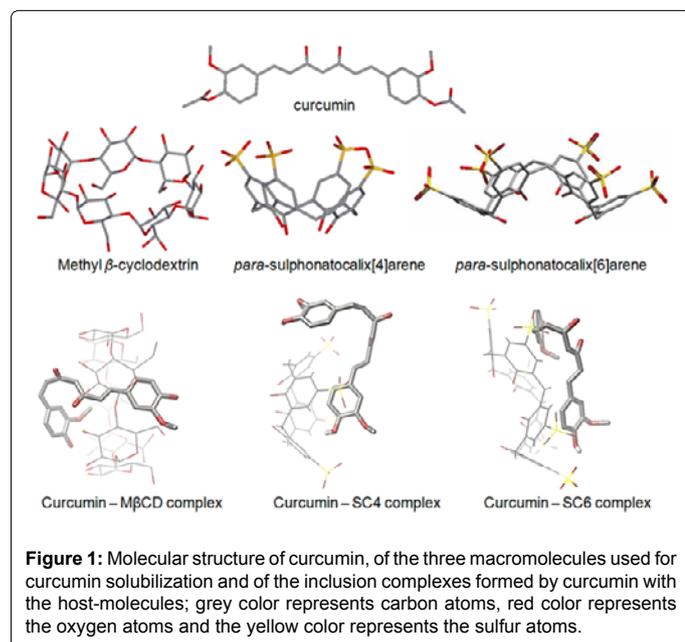


Figure 1: Molecular structure of curcumin, of the three macromolecules used for curcumin solubilization and of the inclusion complexes formed by curcumin with the host-molecules; grey color represents carbon atoms, red color represents the oxygen atoms and the yellow color represents the sulfur atoms.

Complex	Size average (nm)	Polydispersity Index
Curcumin-M β CD	45	0,33
Curcumin-SC4	68	0,42
Curcumin-SC6	75	0,31

Table 1: Characterization of curcumin-macromolecule complexes by dynamic light scattering; average of three runs, rounded to the nearest number.

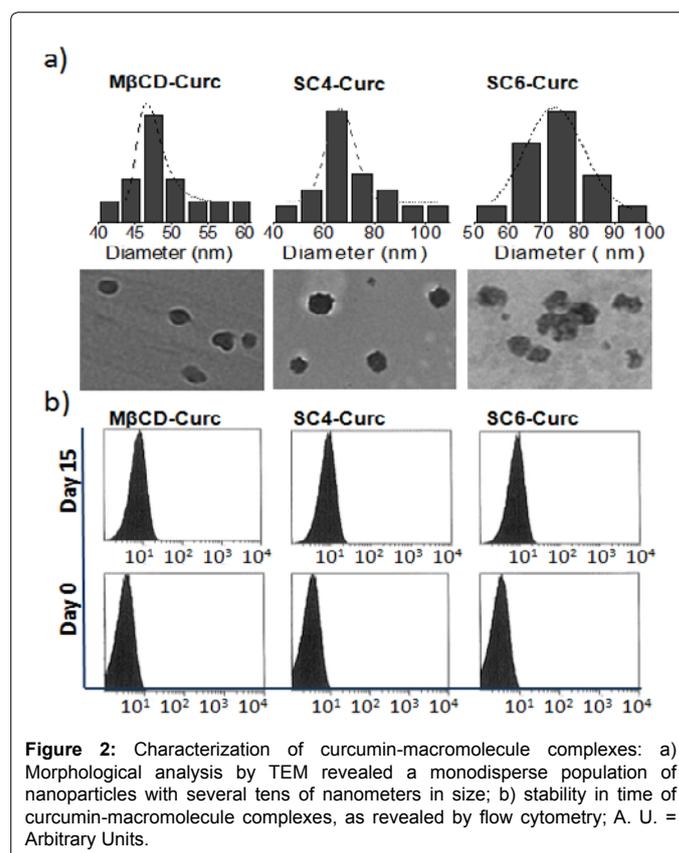


Figure 2: Characterization of curcumin-macromolecule complexes: a) Morphological analysis by TEM revealed a monodisperse population of nanoparticles with several tens of nanometers in size; b) stability in time of curcumin-macromolecule complexes, as revealed by flow cytometry; A. U. = Arbitrary Units.

was partly encapsulated by π - π stacking with one of the phenol groups, and by hydrogen bonds with the sulphonate groups of the host; the other moiety of the molecule is free of constraints. In a similar way SC6 formed a complex with 2 curcumin molecules, one at the upper rim of the calix(6)arene, the other one at the lower rim. Both guest molecules are stabilized by H-bonds interactions with the hydroxyl and sulphonate groups of the host and by π - π stacking interactions of two phenolic rings with the calix(6)arene macrocycle. Given the flexibility of the curcumin, but also of the macromolecules, other structures for the complexes are possible.

Characterization of curcumin-macromolecule complexes

The size of the curcumin-macromolecule complexes was estimated by dynamic light scattering and was found to be of 45 nm for M β CD-curcumin and around 70 nm for SC4/SC6-curcumin (Table 1). The morphology of the curcumin-macromolecule complexes was determined by transmission electron microscopy. Monodispersed nanoparticles of a diameter between 45 and 75 nm were characteristic for the three complexes (Figure 2a). Adapted flow cytometry analysis at different time intervals proved that the size of the nanoparticles diminished slightly in time (several nm), but they remain monodisperse for more than 15 days (Figure 2b).

Post-mortem AD brain tissue

The affinity of curcumin nanocarriers for the amyloid deposits was tested on *post-mortem* brain samples of three AD patients. The amyloid pathology of the cases chosen for the study was confirmed by immunohistochemistry with an antibody frequently used for the detection of the A β deposits, the 6F3D antibody. The cases presented numerous amyloid deposits, from diffuse deposits (an early stage of the senile plaque) to mature senile plaques (Duyckaerts 1990) (Figure 3).

Affinity of curcumin-nanocarriers for amyloid deposits in human AD brains

Numerous amyloid deposits were specifically labeled by the three curcumin nanocarriers. The specificity was confirmed by the double labeling with 6F3D antibody (directed against the A β deposits). The senile plaques were strongly labeled by the curcumin nanocarriers (Figure 4). The nanocarriers also labeled 65% of the diffuse deposits.

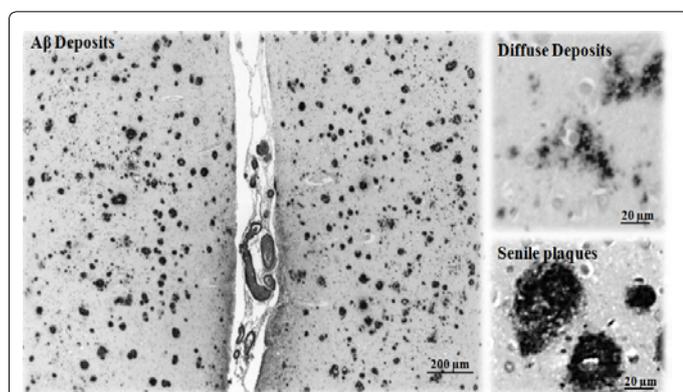


Figure 3: Micrographs of a *post-mortem* superior temporal cortex sample of an AD patient revealed by 6F3D anti-A β antibody: numerous senile plaques and diffuse deposits are present in the cortical area.

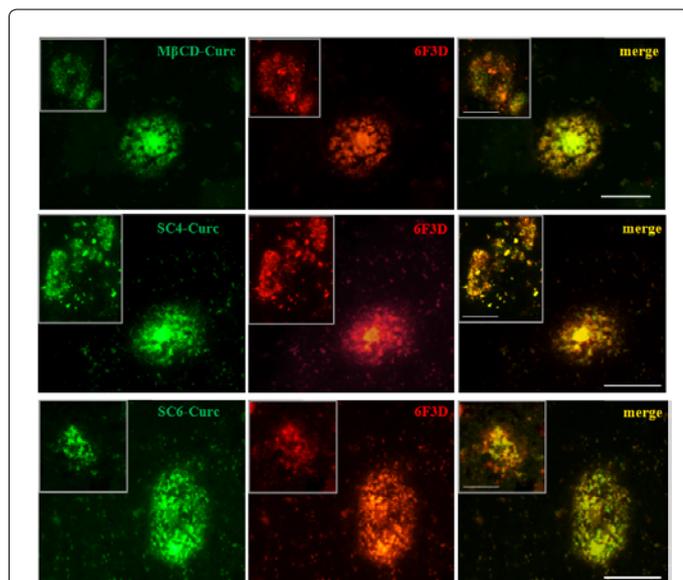


Figure 4: Affinity of curcumin-nanocarriers for amyloid deposits; curcumin labeling in green, 6F3D labeling (specific for A β deposits) in red and the colocalization of the labeling, on the merged image, in yellow; the insert shows the labeling of the diffuse deposits; scale bar =50 μ m.

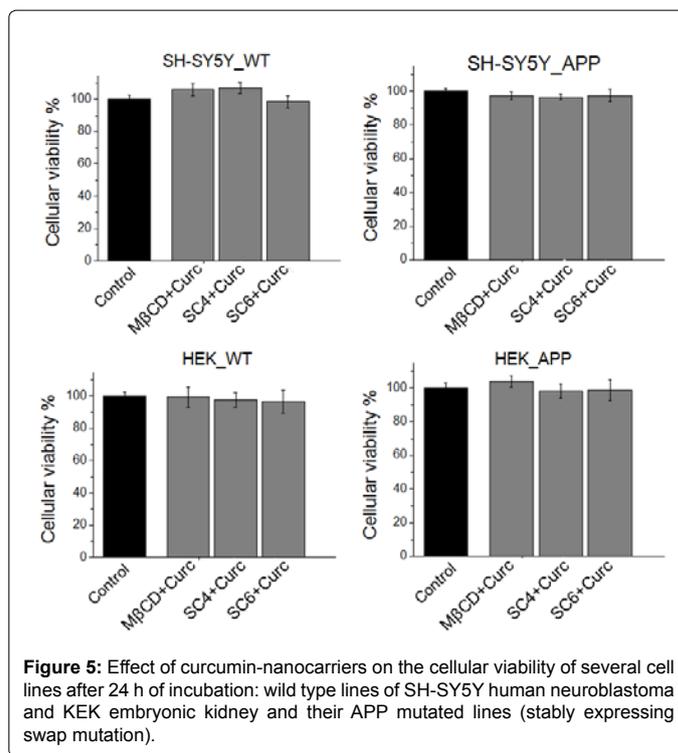


Figure 5: Effect of curcumin-nanocarriers on the cellular viability of several cell lines after 24 h of incubation: wild type lines of SH-SY5Y human neuroblastoma and HEK embryonic kidney and their APP mutated lines (stably expressing swap mutation).

The macromolecules alone were not fluorescent and showed no labeling of the deposits (results not shown).

Biocompatibility of curcumin-complexes as tracers for the amyloid deposits

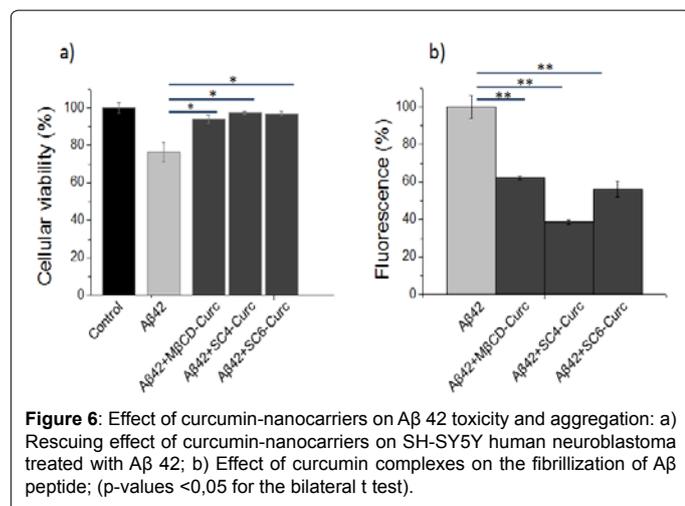
The cytotoxicity of M β CD, SC4 and of SC6 and of the curcumin-nanocarriers formed with these three macromolecules was evaluated by MTT test on human neuroblastoma SH-SY5Y and embryonic kidney HEK cell, wild type and APP (stably overexpressing hAPP bearing the Swedish mutation). Neither the macromolecules alone (results not shown) nor the three curcumin-nanocarriers showed a toxic effect (Figure 5).

Effect on A β toxicity and aggregation

The toxic effect of A β 42 peptide in the presence of curcumin-nanocarriers was evaluated on SH-SY5Y wild type neuroblastoma cells, over 24 h of incubation. The three nanocarriers showed a significant rescuing effect: the viability of cells submitted to A β 42 was increased from 76% to 93% by M β CD-Curc, 97% by SC4-Curc and 96% by SC6-Curc (Figure 6a). The link between the rescuing effect of curcumin-nanocarriers and the ability of curcumin to impede peptide aggregation was evaluated with a thioflavin T assay. The three curcumin-nanocarriers showed a strong inhibitory effect on peptide fibrillization. A relative estimation of the inhibition efficiency showed a reduction of the fibrillar content to: 62% by M β CD-Curc, 39% by SC4-Curc and 56% by SC6-Curc (Figure 6b).

Discussion

A new delivery system, based on the use of macromolecules for solubilizing curcumin, is presented in this study. Hydrosoluble calix(4) arenes, SC4 and SC6, bearing 4 or 6 aromatic cycles functionalized at the upper rim with sulphonate groups, and M β CD efficiently solubilized curcumin. The curcumin-macromolecule complexes were organized



into nanoparticles of tens of nanometers in diameter, stable in time, for at least 15 days. Within the generated nanocarriers, the ability of curcumin to interact with the amyloid deposits in *post-mortem* brain samples of AD patients was conserved. Both mature senile plaques and diffuse deposits—the kinetic precursor of the senile plaques—were labeled by the three curcumin-nanocarriers. The strong affinity for the mature Aβ deposits and especially for the early aggregates points them as potential radiotracers or MRI markers for early diagnosis in AD. In view of this, several *in vitro* tests proved the biocompatibility of these curcumin nanocarriers: they were shown to be non-toxic on different cells lines, to significantly rescue the toxicity induced by Aβ42 aggregates and to limit the fibrillization of Aβ peptide.

Alzheimer's disease is a frequent form of dementia, Aβ plaques and NFTs playing a major role in the development of this disease. Significant evidences suggest that progressive accumulation of Aβ in limbic and association cortices precedes the neurodegeneration of tau and the cascade of biochemical and cellular modifications in the brain. The development of strategies to detect Aβ pathological changes *in vivo*, in the early stages of AD is essential. Currently, no imaging techniques capable of early detection of AD are available in clinics. The PET tracer [11], Pittsburg Compound (PIB) is employed for clinical diagnosis since 2004, after the first human study [49]. Other ¹⁸F-amyloid tracers have been developed since, but their specificity is questioned. Moreover, these tracers have low affinity for the diffuse deposits, the diagnosis indicated being negative in the absence of senile plaques. Magnetic Resonance Imaging (MRI) is also a powerful tool for clinical and biological imaging able to map structure and function with excellent spatial resolution. Numerous amyloid scintigraphic and magnetic probes have been or are being developed. Still, few are fulfilling all the requirements in order to be employed for clinical diagnosis: bioavailable, non-toxic and target specific.

Numerous studies confirmed the potential of curcumin in the treatment of AD [7,44,50-54] and lately, in early diagnosis [5,10,55-58]. The lack of complete success of curcumin essays resides in its low bioavailability, related to its insolubility in aqueous solutions. High efforts were made to improve its solubility, from chemistry to nanotechnology, either by functionalizing the basic molecule with ionic functions or by conjugation of small molecules (particularly amino acids [59]) or by designing nanoparticles based systems, like liposomes [30,60-62], solid lipid nanoparticles [31,33,63-65], or polymeric nanoparticles [32,44,62,66-70]. These types of nano-formulations are

generally based on the inner encapsulation of curcumin. Formulations designed to solubilize curcumin by conserving its ability to bind Aβ peptide or to target the amyloid deposits are few [30,60] and they concern liposomes incorporating phosphatidyl serine covalently linked with curcumin through the polar head.

Solubilization of curcumin by means of inclusion complexes represents a convenient alternative for increasing curcumin bioavailability concomitantly with preserving it available for targeting and interaction with the environment. Among the various existing drug carriers [35,71,72], cyclodextrins have been almost exclusively used for the solubilization of curcumin [37,41-43]. The stoichiometric ratio between cyclodextrins and curcumin was reported to be 2:1 [37,73], suggesting that each of the two phenolic rings of curcumin would be sheltered by the lipophilic cavity of two cyclodextrins. By controlling the molar concentration of each component, a complex ratio of 1:1 cyclodextrin/curcumin can be obtained [38,41]. Such curcumin-cyclodextrin complexes behave like a lipophilic supramolecule, being able to self-assemble into vesicular nanostructures in which curcumin is believed to be confined at the interior vesicle [40]. The potential of cyclodextrin-solubilized curcuminoids in the treatment of AD was already evaluated by the group of Quitschke et al.: by using 2-hydroxypropyl-glycylcyclodextrin, they showed that cyclodextrin-solubilized curcuminoids are able to lower the amyloid plaque load in AD transgenic mice [43]. The study focused mainly on the metabolism of curcumin and curcuminoids. But could such formulations preserve the targeting ability of curcumin? This question was not examined before, to our knowledge.

In this study, we have proved that MβCD-curcumin complex could self-assemble into nanoparticles in a way that retains, at least partially, the curcumin at the surface of the nanoparticles. Likewise curcumin-*para*-sulphonato-calix(4)arene and curcumin-*para*-sulphonato-calix(6)arene complexes self-assembled into nanoparticulate systems, with curcumin at the surface of the particles. Several approaches have confirmed the availability of curcumin at the surface of these nanoparticles: the nanoparticles prevented Aβ42 induced toxicity; they were able to interact with the Aβ peptide, reducing its aggregation and they showed high affinity for the amyloid deposits, strongly labeling not only the senile plaques but also the diffuse deposits of Aβ on brain tissue of *post-mortem* AD patients.

The potential of such curcumin-nanocarriers in early diagnosis as well as in the treatment of AD is to be considered as few reports deal with both therapeutic and preventive approach. The use of not only non-toxic, but also pro-active amyloid tracers may offer the possibility of an early diagnosis combined with a subsequent treatment effect, by preventing the toxicity due to Aβ aggregates, limiting the progression of the aggregation and thus, the evolution of the disease.

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