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# Ultrashort Peptide Theranostic Nanoparticles by Microfluidic-Assisted Rapid Solvent Exchange

Ming Ni, Guillaume Tresset, Ciprian Iliescu and Charlotte A. E. Hauser

**Abstract**—Ultrashort peptides (USPs), composed of three to seven amino acids, can self-assemble into nanofibers in pure water. Here, using hydrodynamic focusing and a solvent exchange method on a microfluidic setup, we convert these nanofibers into globular nanoparticles with excellent dimensional control and polydispersity. Thanks to USP nanocarriers' structure, different drugs can be loaded. We used Curcumin as a model drug to evaluate the performance of USP nanocarriers as a novel drug delivery vehicle. These nanoparticles can efficiently cross the cell membrane and possess nonlinear optical properties. Therefore, we envisage USP nanoparticles as promising future theranostic nanocarriers.

**Index Terms**— self-assembling peptides, theranostic nanoparticles, microfluidics.

## I. INTRODUCTION

Peptides are intriguing building blocks for many types of nanostructures, varying from nanofibers to nanorods, nanoribbons, and many more.[1] Therefore, peptide-based nanomaterials have been widely used as tissue engineering scaffolds, [2] biosensors,[3-5] drug delivery vehicles,[6] and bioimaging probes.[7] Peptides can be a viable drug delivery vehicle alternative to lipid or polymeric micelles vesicles,[8, 9] due to their unique characteristic of recognition by biological systems (e.g. Arg-Gly-Asp(RGD) is a well-known integrin-binding ligand for cell adhesion).[10] Ultrashort peptides (USPs) can be synthesized by solid phase or batch synthesis methods.[11] The ability of USPs to overcome the plasma membrane barrier of mammalian cells make them promising candidates as nanocarriers for drug delivery applications.[12]

The classical way of peptide nanoparticles preparation is through bulk mixing.[13] However, using bulk mixing method, due to the kinetics of self-assembly process, the size and polydispersity of nanocarriers cannot be controlled precisely. This aspect subsequently results in a lack of efficiency for drug delivery. Recent developments in microfluidics enabled us to synthesize nanostructured

materials with excellent control of size, composition and morphology.[14] Different other nanomaterials have been generated successfully via microfluidic mixing methods, e.g. chitosan,[15] lipids,[16-18] and poly(lactic-co-glycolic acid) based nanoparticles,[19-21] DNA-based nanoparticles,[22, 23] theranostic lipoplexes,[24] polysulfone,[25, 26] or multiple layer assembly for the synthesis of polymer microcapsules.[27] Reviews on engineering nanoparticles in microfluidic devices for drug delivery applications are available [28-32].

USPs are a novel class of rationally designed peptides that self-assemble to fibrous nanomaterials.[33] The peptides are composed of three to seven amino acids, with the advantage of an easy manufacturing process in large quantities at relatively low costs due their small size. USPs are amphiphilic in nature, consisting of an aliphatic amino acid tail of decreasing hydrophobicity and a hydrophilic head. These peptides self-assemble most likely via parallel-antiparallel  $\alpha$ -helical pair formation and subsequent stacking into fibers that reorganize to  $\beta$ -turn fibrils which condense to helical fiber bundles (33). These fibers are a few nanometer in diameter and at least micrometers but even up to centimeters in length that can further aggregate and form nanofibrous scaffolds in the form of macroscopic hydrogels.[33] USP hydrogels have been used successfully as 3D printing bioinks,[34, 35] wound dressings,[36, 37] tissue engineering[38] and in cancer therapy.[39, 40] These USPs can also be used as optical imaging probes, being visualized by second-harmonic microscopy.[41]

Herein, we report the preparation of self-assembled USP nanoparticles, using a uniform solvent exchange process enhanced by the microfluidics-based hydrodynamic flow focusing method. USP nanoparticles (USPN) were characterized using scanning electron microscopy (SEM) and dynamic light scattering (DLS) techniques. These nanoparticles were further used for curcumin (as a model drug) encapsulation to test their utility as nanocarriers for drug delivery applications. Due to the optical properties previously reported [41], we propose USPNs as a special class of theranostic nanocarriers.

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## II. MATERIALS AND METHODS

### A. Peptides

Ultrashort peptides, Ac-LIVAGD-NH<sub>2</sub> (LD<sub>6</sub>), Ac-LIVAGK-NH<sub>2</sub> (LK<sub>6</sub>), Ac-LIVAGK-C2-Biotin (LK<sub>6</sub>-Biotin), Ac-IVK-NH<sub>2</sub> (IK<sub>3</sub>) were purchased from the American Peptide Company (Sunnyvale, CA, USA) with high purity ( $\geq 95\%$ ). Net peptide content varied between 60% and 90%. Following reagents were purchased from the respective companies: Methanol (JTBaker), ethanol (Fisher) and 1, 1, 1, 3, 3, 3-hexafluoro-2-propanol (HFIP, Merck).

### B. Device Fabrication

The microfluidic device consists of two inlets: A central one for the aqueous peptide solution and a second one for different solvents. Figure 1a shows a detailed layout of the microfluidic setup. The central peptide stream was hydrodynamically focused between two side streams containing solvents. The dimensions of the focusing channel were  $60\mu\text{m}\times 40\mu\text{m}\times 15\mu\text{m}$ . In order to vary the degree of focusing, the flow rate ratio can be easily adjusted by a pressurized pumping system (Fluigent, France) that incorporates pressurized reservoirs and mass flow controllers in a software controlled closed loop.

A detailed fabrication process of the microfluidic device can be found in [42]. Briefly, the structure of the microfluidic device was “printed” on a  $300\mu\text{m}$ -thick silicon wafer using two classical microfabrication processes: photolithography and deep RIE etching. The microfluidic channels were defined from top side of the wafer, while the etch-through holes were performed from the opposite side. The SiO<sub>2</sub> layer was grown (thermal dry oxidation) from the surface of the silicon structure in order to achieve a full hydrophilic surface of the microfluidic channel. The microfluidic channel was closed using a Pyrex glass wafer by anodic bonding. After dicing, Nanoport microfluidic connectors were mounted on the Si surface of the device. An image with the fabricated microfluidic device is presented in Figure 1b. Besides the advantage of the robustness, reusability and good dimensional control of the microfluidic structure, the main advantage of the glass-silicon device is that it allows an “on-site” cleaning of the microfluidic channels.[43] In our case this cleaning was done by using the same procedure, but just filling the reservoirs with N-methyl pyrrolidone (NMP) solution letting it flow through the microfluidic setup, followed by cleaning with isopropyl alcohol (IPA) and deionized (DI) water.

### C. Preparation of ultrashort peptide nanoparticles (USPNs)

The central aqueous peptide (using peptides LD<sub>6</sub>, LK<sub>6</sub> and IK<sub>3</sub>) stream was hydrodynamically focused between the side streams containing solvents (methanol, ethanol or HFIP). To Preparation of ultrashort peptide nanoparticles (USPNs) vary the degree of focusing, the flow rate ratio can easily be adjusted by a pumping system (MFCS-FLEX pumping system, Fluigen, France) that incorporates pressurized reservoirs and mass flow controllers in a software-controlled closed loop. For curcumin encapsulation, LK<sub>6</sub>-biotin was dissolved in pure water at the concentration of 1 mg/mL and this solution was used for the central stream. Curcumin was dissolved in ethanol and then

diluted to 1 mg/mL in 50% ethanol and the solution served as side streams.

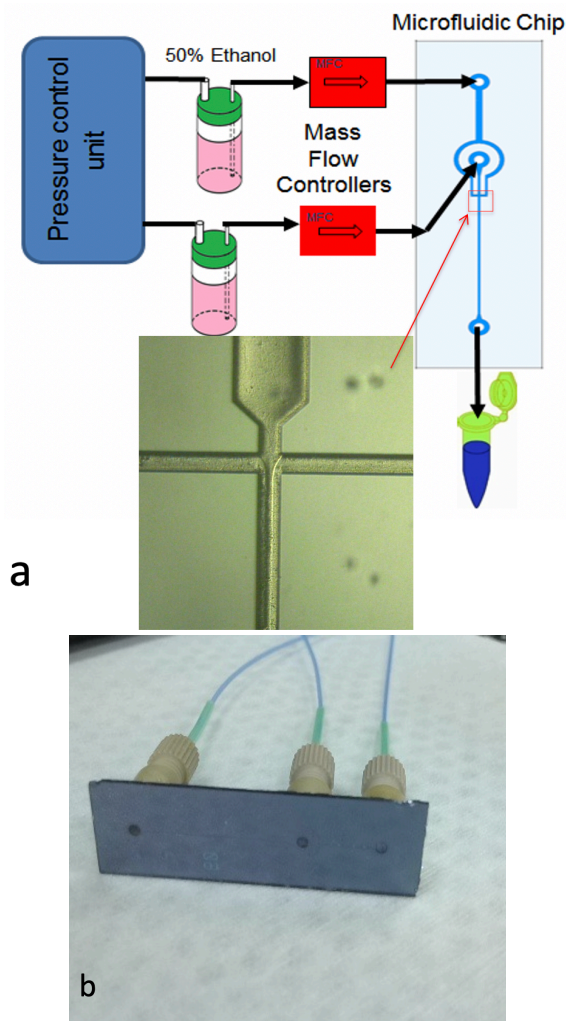


Fig. 1. a) Detailed layout of the microfluidic setup used in this work; b) image with the microfluidic chip with the mounted Nanoport connectors.

### D. Characterization of USP nanoparticles

The size and polydispersity (PDI) of USP nanoparticles were measured by dynamic light scattering (DLS) using a Zetasizer model ZS-90 (Malvern, UK). The morphology of the nanoparticles was visualized by a JSM-7400F field emission scanning electronic microscopy (FE-SEM, JEOL, Tokyo, Japan). The stability of these nanoparticles was monitored by DLS over a period of one week.

### E. Cellular uptake of Curcumin-encapsulated USP nanoparticles

HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing high concentration of glucose, supplemented with 10% fetal bovine serum (FBS, Life Technologies) and 1% penicillin/streptomycin solution (Gibco®, Life Technologies). Cells were seeded onto four-well chamber slides (SPL, Korea) at a density of  $2 \times 10^4$  cells/well and cultured overnight. Before adding the nanoparticles, cells were pre-incubated with Opti-MEM (Gibco®, Life

technologies) at 37°C for 30 min. Curcumin-encapsulated USP nanoparticles were first dispersed in Opti-MEM at a peptide concentration of 0.1 mg/mL. These nanoparticles were then incubated with the cells. After 1 h of incubation, cell medium was removed and cells were fixed with 3.7% formaldehyde. To visualize peptides, DyLight™ 488-conjugated NeutrAvidin™ (Pierce) was added to the cells. The cells were also stained with 4,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich). The prepared chamber slides were examined by confocal laser scanning microscopy (CLSM, Zeiss LSM 510 META).

### F. In Vitro Release of Curcumin from USP Nanoparticles

Curcumin-encapsulated USP nanoparticles (0.2 mL), enclosed in Slide-A-Lyzer™ dialysis cassettes (molecular weight cut-off (MWCO) = 3500), were incubated in 100 ml PBS (pH 7.4) at 37°C under mild agitation in a water bath. At predetermined time intervals, 1 mL samples withdrawn from the incubation medium and analyzed for curcumin uptake with fluorescence spectroscopy (Horiba, Japan). Fluorescence intensity of known curcumin concentrations (0, 5, 10, 20, 30 40 ng/mL) was used to establish a standard calibration curve.

## III. RESULTS AND DISCUSSIONS

Initially, Ac-LIVAGK-NH<sub>2</sub> peptide (LK<sub>6</sub>, American Peptide Company, peptide purity=98.8%, peptide content =67.7%) was chosen as the model peptide. The hydrodynamic focusing method for the processing of USPNs by employing a microfluidic device has been previously described [22,42]. It can be seen that the central stream is strongly focused by two side streams (**Fig. 1a**). In order to achieve uniform sized USPNs, the concentration on the central stream (1 mg/mL LK<sub>6</sub> peptide solution) was kept constant while for the side streams different types of solvents were tested. Several chemical reagents were chosen, including methanol, 50/50 ethanol/water and hexafluoroisopropanol (HFIP). The hydrodynamic diameter of USPNs and the size distribution -polydispersity index (PDI) were measured using DLS, (Malvern, UK). **Fig. 2** shows that among three different solvents, 50/50 ethanol/water solvent mixture gave nanoparticles with a single peak. Therefore, we focused for further experiments on the ethanol-water mixture as the solvent for the side stream.

Another important aspect is the *potential of fine-tuning of the hydrodynamic diameter of USPNs*. In this direction, the influence of the concentration of the solvent and the concentration of the peptide solution was studied. **Fig. 3a and 3b** show the effect of ethanol concentration of the hydrodynamic diameter distribution of USPNs. First, the concentration of LK<sub>6</sub> (1 mg/mL) in the central stream was kept constant and different ethanol concentrations (from 10 vol% up to 80 vol%) in the side streams were tested. It was found that the optimal value, in terms of USPNs size and distribution, of the ethanol concentration is between 50 to 60%(vol). To investigate the effect of peptide concentration on the particle size and size distribution of USPNs (**Fig. 3c and 3d**), the ethanol concentration in the side stream was kept constant at 50 vol% while the peptide concentration (LK<sub>6</sub>) was varied from 10 µg/mL to 2 mg/mL. It was found that the size of the

nanoparticles became smaller as the concentration of the peptide decreased. At the same time, we still achieved uniform-sized particles (PDI<0.2).

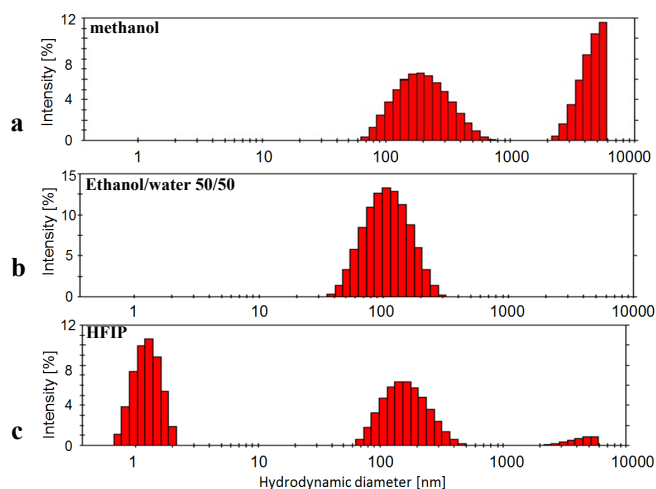


Fig. 2. The distribution of the hydrodynamic diameter (DLS measurement): (a) methanol; (b) 50/50 ethanol/water; (c) 1, 1, 1, 3, 3, 3-hexafluoro-2-propanol (HFIP)

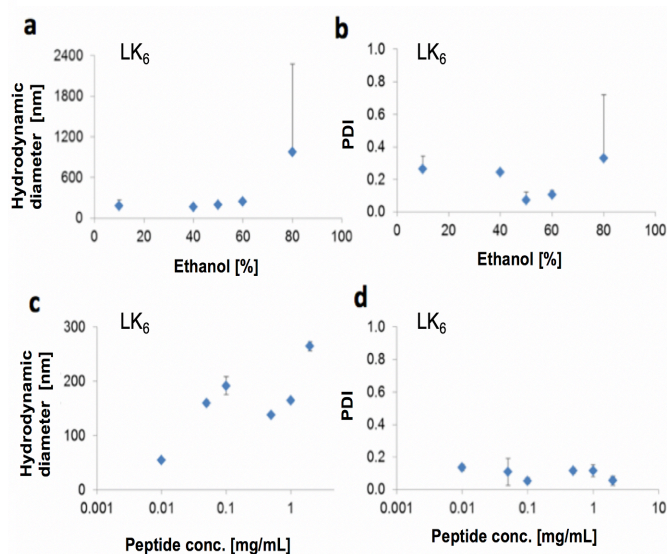


Fig. 3. Effect of ethanol and peptide (LK<sub>6</sub>) concentration on the particle size and size distribution of USPNs. The size distribution was determined by using DLS. Effect of ethanol concentration on the (a) size and (b) polydispersity of USPNs. Effect of peptide concentration on the (c) size and (d) polydispersity of USPNs.

Furthermore, *the effect of peptide composition and chain length* on the formation of USPNs was investigated. Three different peptides were chosen: Two hexamer peptides LK<sub>6</sub> and LD<sub>6</sub> and a trimer peptide IK<sub>3</sub>. All these three peptides, LK<sub>6</sub>, LD<sub>6</sub> and IK<sub>3</sub>, belong to the same class of ultrashort self-assembling peptides (33). In **Fig. 4**, both scanning electron microscopy (SEM) images and DLS measurements showed uniform sized particles. Different to the spherical morphology of the peptide LK<sub>6</sub> and LD<sub>6</sub> particles, peptide IK<sub>3</sub> particles showed an anisotropic particle morphology. It is another signature that peptides were first assembled into nanofibers. [33]

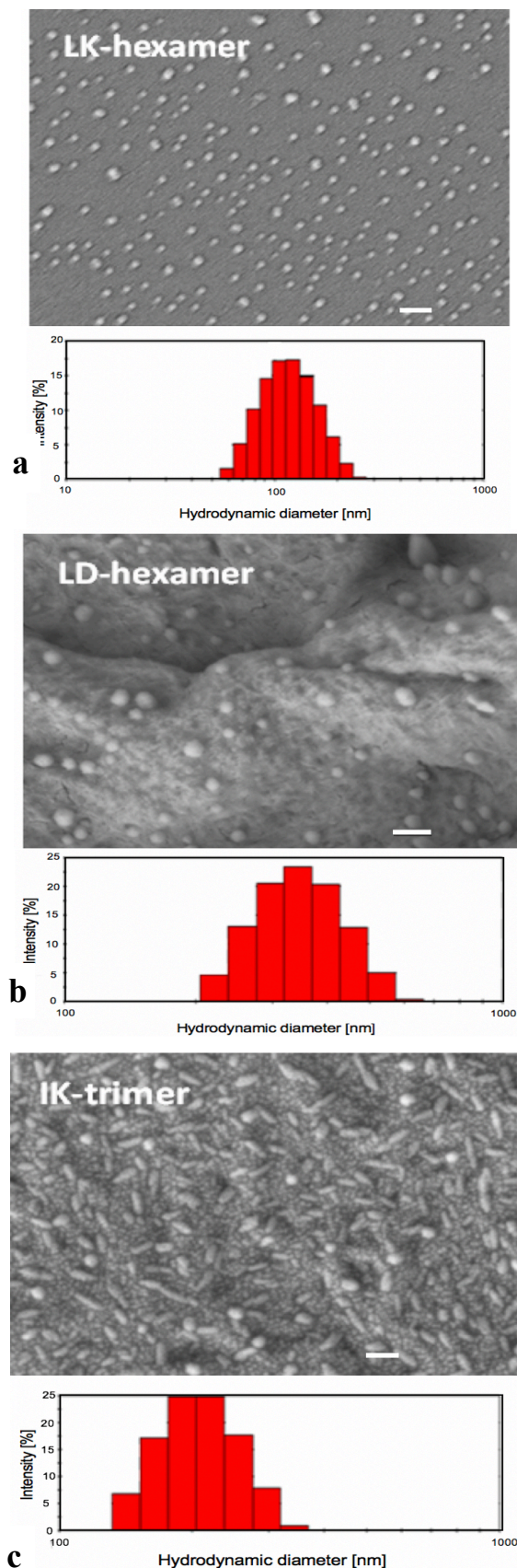


Fig. 4. Comparison of particle size and size distribution of USPNs (SEM pictures +DLS distribution) prepared from three different ultrashort peptides (a) LK<sub>6</sub>, (b) LD<sub>6</sub>, and (c) IK<sub>3</sub>. Scale bar = 200 nm.

The influence of fluid velocity in the microfluidic channel on the hydrodynamic diameter of the USPN was also investigated. The flow rate ratio peptide (LK<sub>6</sub>)/ ethanol was maintained constant while the total flow rate was increased. The results are summarized in **Table 1**, and it can be noticed that no relevant variations were observed. Moreover, we tried to vary the flow rate ratio by keeping the peptide flow rate constant at 3  $\mu\text{L}/\text{min}$  and changing the flow rate of the solvent (values of 12  $\mu\text{L}/\text{min}$ , 15  $\mu\text{L}/\text{min}$ , 18  $\mu\text{L}/\text{min}$ , 21  $\mu\text{L}/\text{min}$  and 24  $\mu\text{L}/\text{min}$ ). The results are summarized in **Table 2** and show a relatively constant value of the hydrodynamic diameter of the nanoparticle with an acceptable value of the polydispersity.

TABLE I  
THE VARIATION OF THE HYDRODYNAMIC DIAMETER OF THE PEPTIDE (LK<sub>6</sub>) NANOPARTICLES WITH THE TOTAL FLOW RATE (VELOCITY OF THE FLUID) AT CONSTANT FLOW RATE RATIO

Flow rate ratio (peptide/ethanol)	Hydrodynamic diameter	PDI
3:12	162.9 $\pm$ 0.98	0.016
4:16	166.7 $\pm$ 2.01	0.034
5:20	166.2 $\pm$ 1.21	0.021

TABLE II  
THE VARIATION OF THE HYDRODYNAMIC DIAMETER OF THE USP<sub>N</sub> (LK<sub>6</sub>) WITH THE TOTAL FLOW RATE RATIO

Flow rate ratio (peptide/ethanol)	Hydrodynamic diameter	PDI
3:12	162.9 $\pm$ 0.98	0.016
3:15	163.7 $\pm$ 1.12	0.034
3:18	164.1 $\pm$ 1.21	0.021
3:21	155.4 $\pm$ 0.61	0.020
3:24	155.5 $\pm$ 2,56	0.023

To explore the utility of USP<sub>N</sub>s as drug carriers, curcumin was encapsulated into USP<sub>N</sub>s using the microfluidic flow-focusing method. Curcumin-loaded USP<sub>N</sub>s were incubated with HeLa cells and the cellular uptake of USP<sub>N</sub>s was visualized by confocal laser scanning microscopy (CLSM). After 1 h incubation, the cells were fixed with 3.7% formaldehyde and afterwards the biotin-labelled peptides were stained with NeutrAvidin Dylight® 488. Fluorescence micrographs (**Fig. 5**) clearly demonstrated the cellular uptake of curcumin-containing peptide nanoparticles by HeLa cells. It can also be seen that the curcumin-containing peptide nanoparticles were present in the cytoplasm (**Fig. 5**). As a control, we also tested the uptake of curcumin as a free drug (data not shown). Curcumin was found in the cell nucleus but not in the cytoplasm (the mechanism for nucleolar homing of curcumin can be found in [44]). These results clearly show the potential of using USP<sub>N</sub>s as drug carriers. Curcumin is a promising drug candidate for the treatment of neurodegenerative diseases such as Parkinson's and Alzheimer's diseases due to its neuroprotective effects and ability to cross the blood-brain barrier. [45] However, curcumin suffers from low half-life in the

blood stream (5-10 minutes). Therefore, it is essential to use the concept of “controlled release” to deliver curcumin in a sustainable way. Hence, curcumin-containing USPNs may be used to repair damaged brain tissue.

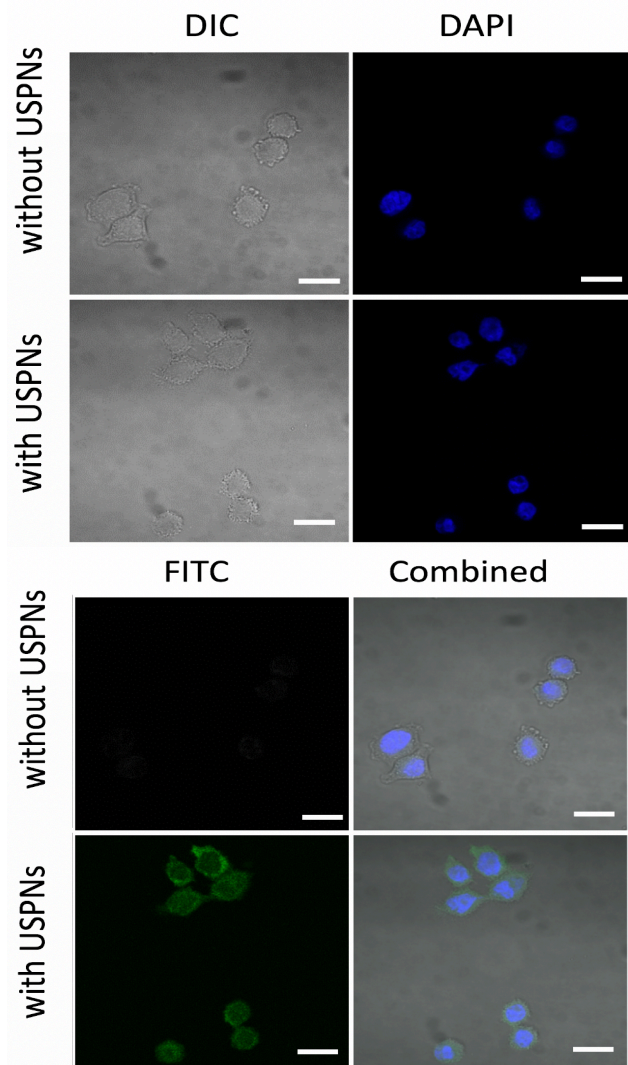


Fig. 5. Cellular uptake of curcumin-loaded LK<sub>6</sub>-biotin nanoparticles. Upper panel: HeLa cells exposed to normal medium without USPNs (control); Lower panel: HeLa cells exposed to curcumin-loaded LK<sub>6</sub>-biotin nanoparticles containing medium. Peptides in HeLa cells displayed green color (fluorescence); cell nucleus displayed blue color (DAPI). Scale bars: 20μm

The *in vitro* release of curcumin from the USP nanoparticles was further studied. Curcumin-encapsulated USP nanoparticles (0.2 mL), enclosed in Slide-A-Lyzer™ dialysis cassettes (molecular weight cut-off (MWCO) = 3500), were incubated in 100 ml PBS (pH 7.4) at 37°C under mild agitation in a water bath. At predetermined time intervals, 1 mL samples were withdrawn from the incubation medium and analyzed for curcumin by fluorescence spectroscopy (Horiba, Japan). Fluorescence intensity of known curcumin concentrations (0 to 40 ng/mL) was used to establish a standard calibration curve. We have monitored the *in vitro* curcumin release from USPNs as shown in Fig. 6. Most of the drug was released from the USPNs after 24 h incubation (much longer time than its half-life in the blood stream).

We found that even after six days, more than 30% of the drug was still trapped inside the USPNs. These results are encouraging, because we expect that the USPNs will be degraded inside the cells, which will eventually lead to 100% drug release. We further monitored the particle size and distribution of USPNs over a period of a week and no significant variation (Table 3) in the USPNs size and distribution was observed. Although we did not study the stability of USPNs over a longer period of time, however, these results are encouraging to formulate a controlled-release form of curcumin for therapeutic applications in the near future. In a previous work, we demonstrated that USP particles are second harmonic-active and can be visualized by second harmonic generation microscopy.[41] These unique nonlinear optical properties encourage the use of USPNs as potential theranostic agents (imaging and therapeutics).

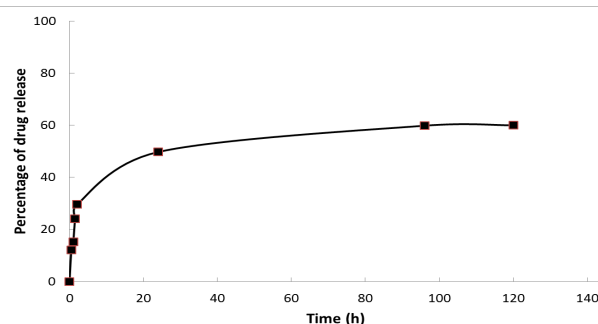


Fig. 6. In vitro release of Curcumin from USPN

TABLE III  
THE HYDRODYNAMIC DIAMETER AND DISTRIBUTION OF CURCUMIN-LOADED USPNs (AVERAGE ± STANDARD DEVIATION, N=3).

Day	Hydrodynamic diameter	PDI
1	68.35 ± 0.30	0.109±0.026
5	70.08 ± 2.73	0.237±0.043
6	71.15 ± 3.46	0.285±0.103
7	74.31 ± 0.59	0.196±0.013

#### IV. CONCLUSIONS

In conclusion, we have successfully prepared uniformly sized USPNs using the microfluidic hydrodynamic focusing method and the self-assembly characteristics of USP. These nanoparticles are small in size (< 100 nm) and show good uniformity (PDI < 0.2). The preliminary *in vitro* curcumin release studies showed that curcumin can be successfully encapsulated into USPNs and slowly released from USPNs. Curcumin-loaded USPNs are stable for at least one week and can be uptaken by mammalian cells. USPNs are also second harmonic-active, which makes them promising candidates as theranostic nanocarriers.

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