Pharmaceutical Formulation and Characterization of Dipeptide Nanotubes for Drug Delivery Applications

Simon L. Porter, Sophie M. Coulter, Sreekanth Pentlavalli, and Garry Laverty*

Peptide nanotubes are promising materials for a variety of biomedical applications with ultrashort (<7 amino acids) forms providing particular promise for clinical translation. The manufacture of peptide nanotubes has, however, been associated with toxic organic solvents restricting clinical use. The purpose of this work is to formulate dipeptide nanotubes using mild techniques easily translated to industrial upscale and to characterize their physiochemical and biological properties. Phenylalanine-phenylalanine variants can be successfully formulated using distilled water as demonstrated here. Formulations are homogenous in shape (tubular), with apparent size (50–260 nm) and a zeta potential of up to +30 mV. L-(H$_2$N-FF-COOH), and D-enantiomers (H$_2$N-ff-COOH) demonstrate no toxicity against glioblastoma cells and are explored for ability to deliver a model hydrophilic molecule, sodium fluorescein, at pH 5.5 (tumor) and 7.4 (physiological). Peptide nanotubes loaded with >85% sodium fluorescein, demonstrate burst release characteristics, fitting the Weibull model of drug release. This research provides important data contributing to the pharmaceutical formulation of peptide nanotubes as drug delivery platforms for hydrophilic drugs.

1. Introduction

Peptide nanotubes are an exciting, innovative platform currently being researched for a variety of applications, including electronics,[1] sensors,[2] alternative energy harvesters,[3] and biomedical materials.[4] Nanoparticle formulations, including peptide nanotubes, can offer several unique advantages as drug delivery platforms. The benefits of nanoparticles include: i) increased stability and increased resistance to clearance from the body, ii) delivery of higher therapeutic drug concentrations, iii) passive targeting of nanoparticles to solid tumors through the enhanced permeability and retention effect, iv) permeation of biological barriers that are inaccessible to certain “naked” drug molecules.[5,6] A multitude of nanosized systems, including carbon, polymeric, and metallic variants, have been explored for use as drug delivery platforms, however peptides possess several significant advantages.[7] For example, peptides allow for tunable physicochemical properties due to the varying nature of their amino acid R-chemical functional group. This can be exploited, for example, to load a diverse array of molecules and drugs, including chemotherapies.[8–10]

Due to the chemical versatility of the peptide motif and their amino acid building blocks, the primary peptide sequence and molecular structure has the potential to be altered to target specific molecular recognition sites, cells, tissues, and/or disease states and to incorporate a wide variety of hydrophilic and hydrophobic drugs with high loading capacities.[11–13] The tunable nature of the peptide sequence also enables tailoring of biodegradation, biocompatibility, and immunogenicity for required purpose.[13,14] Of particular interest are ultrashort variants, such as dipeptide nanotubes composed of two amino acid subunits, which are well documented for their ability to self-assemble.[15,16] Utilizing the minimum number of subunits required to achieve self-assembly facilitates cheap manufacture thereby increasing the potential for clinical translation and therefore phenylalanine-phenylalanine nanotubes form the focus of this investigation. It has been widely established that the ability of a dipeptide motif to self-assemble into nanotube architectures is largely dependent on the presence of two hydrophobic amino residues within the peptide primary sequence.[17] Intermolecular forces, for example a combination of π-π interactions, van der Waals forces, and hydrogen bonding, provide sufficient energy to form the nanotube shape with a central solvent-filled channel.[19] For dipeptides this hydrophilic channel is the largest with phenylalanine-phenylalanine motifs (van der Waals diameter 10 Å), making it an attractive selection as a model motif with the potential to load increased quantity of hydrophilic drugs.[17]

As therapeutics, peptide nanotubes of various size have been mainly studied for their antibacterial properties,[20] with dipeptide variants as well as longer sequences, demonstrating inherent activity due to their ability to interact with cellular membranes causing depolarization.[21] Our research group recently demonstrated dipeptide nanotubes to be highly active...
against biofilm forms of infection implicated in increased tolerance of bacteria to antibiotics, providing a possible solution to alleviate hospital superbug infections.\[22\] Peptide nanotubes represent a novel strategy to deliver drugs to specific sites across the body and as such are receiving increased attention for this application. Their size, lying within the nanometer range, and shape (tubular structure) make them optimal molecules for interacting with and crossing biological barriers, for example the cell membrane. A vast number of drugs can be readily enclosed within these nanotubes and therefore they can be potentially utilized to transport drugs into the intracellular space to target cell organelles. There are several advantages of nanotube shape over other nanoarchitectures (e.g., nanospheres, nanocubes, liposomes). The advantage of nanotube shape for cancer delivery has been established for other synthetic-based materials, for example carbon-based nanotubes.\[23,24\] However, the inherent advantages of a peptide-based platform (chemically versatile, nontoxic in nanotube form/breakdown products, ease of manufacture, improved biocompatibility) mean clinical translation of a peptide technology is likely more feasible resulting in significant benefits to patients and their carers.\[25\] Nanotubular structures have been shown to penetrate into intracellular environments more freely than other nanostructures,\[24\] thereby offering enhanced therapeutic strategies for delivering drugs to specific organelles, for example the nucleus in DNA tumor targeting.\[26\]

Due to a high aspect ratio (length:diameter), tubular particles have a larger surface area in contact with target cells and therefore adhere better to cells and are internalized more efficiently, demonstrating improved transport and delivery throughout the body. This enhanced surface area of nanotubes improves drug loading efficiency meaning more drug can reach target sites at a lower therapeutically administered dose. This should improve drug efficacy at lower concentrations and reduces likelihood of drug-induced side effects at equivalent doses.\[24\] Previous research has shown tubular nanoparticles to be engulfed less readily by macrophages (phagocytosis), meaning improved circulation times and enhanced delivery to site of action.\[23\] Particles within the nanometer size range have the potential to overcome several biological barriers, including cell membranes, thus expanding the library of drugs that are available to treat a wide variety disease. One of the major biological barriers that exists is the tight junctions between endothelial cells in the blood–brain barrier. Very small molecules and some lipid soluble drugs are capable of passively entering the central nervous system after systemic intravenous administration. A variety of nanoparticles have demonstrated success in overcoming the blood–brain barrier via passive or active transport methods.\[27\]

Nanoparticles have to bypass the reticuloendothelial system and avoid the clearance by the spleen and liver before they reach the targeted disease sites. The size of the particles can play an important role in accumulation at diseased sites. The nanoparticles used in nanomedicine typically range from 20 to 200 nm since nanoparticles larger than 200 nm are mechanically filtered in the spleen, while those smaller than 100 nm leave the blood vessels through fenestrations in the endothelial lining. Microparticles (≥1000 nm) are cleared by Kupffer cells in liver or physically trapped in the capillary beds. For particles larger than 200 nm, deformability is required in order to navigate through the liver and spleen. Smaller nanoparticles are generally more efficient at entering cells, with one study reporting that poly(lactic-co-glycolic acid) nanoparticles of size 100 nm had a 2.5-fold increase in uptake into Caco-2 cells than particles of 1000 nm.\[28\] Although increased cellular uptake is generally favorable, particles that are too small will avoid renal filtration as well as opsonization by the reticuloendothelial system resulting in organ accumulation. This is demonstrated by the biodistribution of gold nanoparticles in vivo, with 10 nm nanoparticles showing greater retention in liver and spleen 24 h post injection versus 50, 100, and 250 nm nanoparticles.\[29\] The in vivo hydrodynamic diameter is often larger than the in vitro diameter due to adsorption or opsonization by serum proteins. Nanoparticles greater than 200 nm may suffer from a greater degree of opsonization.\[30\] An in vivo hydrodynamic diameter ≤5.5 nm is widely considered to be optimal in order to permit complete elimination of nanoparticles from the body.\[31,32\] Depending on the desired end target site and considering these various factors, the optimum size range for nanoparticles in drug delivery applications lies in the 10–200 nm range. Preparation and self-assembly of peptide nanotubes by a process of solvent induction has proven the most common method of formulation. However, one major factor that limits this approach to pharmaceuticals is the use of organic solvents, e.g., 1,1,1,3,3,3-hexafluoro-2-propanol (HFP), as a dissolution medium to fully dissolve the peptide as part of their initial formulation with the addition of a polar solvent, most commonly water, triggering self-assembly of nanotubular structures.\[33\] The toxicity of organic solvents lowers the acceptability of such formulations to the pharmaceutical industry, regulators, and patients and therefore it is a significant barrier to the clinical translation of this technology.\[34,35\] A focus of this work is to improve upon existing commonly employed methods of formulation to create nanotube structures with homogenous physicochemical (shape, size, zeta potential) and biological (cell toxicity) properties, using biocompatible water-based solvent systems and testing their ability to load and deliver a model hydrophilic drug (sodium fluorescein). Dip peptide nanotubes, as discussed, display numerous advantages for clinical translation and therefore form the focus of this investigation.

2. Experimental

2.1. Materials

Fluorenylmethoxycarbonyl (Fmoc) protected amino acids, Wang resin, 4-methylbenzhydrylamine (MBHA) rink amide resin, 2-(1H-benzotriazol-1-yl)-1,3,3,3-tetramethyluronium hexafluorophosphate, hexafluorophosphate benzotriazole tetramethyl uronium (HBTU), and Hydroxybenzotriazole (HOBt) were purchased from Novabiochem (Hertfordshire, UK). Thioanisole, N,N-diisopropylethylamine, trisopropylsilane (TIPS), deuterated dimethylsulfoxide (d6-DSO), tripyan blue, sodium fluorescein, phosphate buffered saline (PBS), and acetic acid buffer were purchased from Sigma-Aldrich (Gillingham, UK). UltraPure DNase/RNase-free sterile distilled water was purchased from Gibco.
2.2. Peptide Synthesis, Purity, and Identification

Peptides (H$_2$N-FF-COOH, H$_2$N-ff-COOH and H$_2$N-FF-NH$_2$) were synthesized using standard Fmoc solid-phase peptide synthesis methods as previously employed by our group.[36,37] Wang resin was utilized for carboxylic-acid-terminated dipeptides and MBHA rink amide resin for amide-terminated peptide. All reactions took place under agitation using bubbled nitrogen gas. After initial swelling of resin in DMF, Fmoc protecting groups were removed from the resin, as well as from the amine terminus of subsequent amino acids, using 20% v/v piperidine to break the base-labile bond. For coupling reactions, HBTU was used to activate the amino acids for conjugation with TIPS added to scavenge carbocyanation species. To remove the completed and deprotected peptide from the solid resin support, the peptide was cleaved using a mixture of TFA: TIPS: thioanisole (95:2.5:2.5 v/v) cleavage cocktail in a glass round bottom flask, stirred for 2 h and 30 min. After filtering through a Buchner funnel to remove the solid resin, the majority of the TFA from the cleavage cocktail was removed via rotary evaporation and chilled diethyl ether (=–20 °C) was added to precipitate the peptide. After removal of diethyl ether by evaporation under nitrogen gas, the final product was lyophilized by placement in a freeze dryer (Edwards Modulyo F101-01-000) overnight. Crude product was dissolved in ethyl acetate, washed with 1 mM HCl (3 × 50 mL) and water (3 × 50 mL) and dried over anhydrous magnesium sulfate (MgSO$_4$). Lyophilized acetate salts of H$_2$N-FF-COOH, H$_2$N-ff-COOH, and H$_2$N-FF-NH$_2$ were identified by 1H NMR spectra using a Bruker Ultrashield Plus 400 MHz (Bruker, Coventry, UK) with a minimum of 64 scans (Figures S1–S3, Supporting Information). Samples were prepared by dissolving peptide in deuterated DMSO (d$_6$-DMSO). Mass spectra (Table S1, Table S2, Supporting Information) were obtained by electrospray mass spectrometry (Thermo Finnigan LCQ Deca ion trap, Thermo Fisher Scientific, Waltham, USA). Peptide purity was confirmed as greater than 95% by reverse phase-HPLC (Agilent 1260 series, Agilent Technologies Ltd, Cork, Ireland), using a Gemini C$_8$ column (250 × 4.6 mm) with a flow rate of 1 mLmin$^{-1}$ and UV detection at 220 nm. Samples were dissolved in HPLC grade water and 10 µL was injected (Figure S4, Supporting Information). A gradient elution was used with solvent A, 99.9% H$_2$O, 0.1% TFA and solvent B 99.9% ACN, 0.1% TFA. The gradient began at 10% solvent A and 90% solvent B at time zero and ended at 100% B after 20 min.

2.3. Formulation of Dipeptide Nanotubes

Nonhomogeneous peptide self-assembly will slowly and spontaneously occur when H$_2$N-FF-COOH, H$_2$N-ff-COOH, and H$_2$N-FF-NH$_2$ are dissolved in UltraPure DNase/RNase-free sterile distilled water at room temperature. To allow dipeptides to fully dissolve without the use of organic solvents, and to improve formation of homogenous nanotubes, the peptide solutions were heated with Eppendorfs to 65 °C for 30 min using a Grant Boekel heating block (Boekel Scientific, Feasterville, PA, USA) and then allowed to self-assemble at room temperature for 24 h (Table 1). This method was similar to that employed by Kim et al.[38] but at lower temperatures and without the use of sonication, and the Shelnutt group who utilized temperatures of 65 °C to form peptide nanotube-platinum nanoparticle composites for catalytic applications.[39] Dipeptide nanotube suspensions were titrated to pH 7.4 ± 0.2 with approximately 20 µL of 1 m NaOH solution to ensure pH was constant for further analysis, e.g., cell studies. Fresh formulations were prepared for each experiment and used with 24 h of step 5 (Table 1).

2.4. Microscopy

Scanning electron microscope (SEM) images were produced on a FEI Quanta-250 microscope (Thermo Fisher Scientific, Oregon, USA) to provide an indication of micro/nanoarchitectures of formulated peptides. Ten microliters of peptide nanotube formulation was placed onto a carbon based, electrically conductive, double-sided adhesive disc (Agar Scientific, Essex, UK) and subsequently fixed to the SEM sample fixture. SEM samples were sputtered with a thin layer (8 nm) of gold prior to imaging under vacuum. Images were captured at 2000–20 000× magnification at 20.0 kV and using Everhart-Thorley detector mode settings.[32] Fluorescence analysis was performed on a FLUOstar Omega plate reader (BMG Labtech, Ortenberg, Germany). Live/Dead staining, optical LN229 cell images and visualization of fluorescent model drug loading were taken on fluorescence microscope (EVOS FL microscope, Thermo Fisher Scientific, Waltham, Massachusetts, USA) and processed using ImageJ software version 1.8 (National Institutes of Health, Bethesda, Maryland, USA).

| Step | Formulation method |
|------|--------------------|
| 1    | Quantity of appropriate dipeptide weighed (e.g., 10 mg). |
| 2    | UltraPure DNase/RNase-free sterile distilled water added to dipeptide to provide required concentration (e.g., 1 – 10 mg mL$^{-1}$). Vortex for 30 s. |
| 3    | Heat dipeptide-water mixture at 65 °C for 30 min. |
| 4    | Allow to self-assemble at room temperature for 24 h. |
| 5    | pH of dipeptide nanotube suspensions titrated to pH 7 ± 0.2 with 1 m NaOH solution. |
2.5. Circular Dichroism

Peptide nanotubes were formulated as described in Table 1. Samples were tested at 0.04 mg mL−1 concentration in order to avoid excessive absorbance and analyzed at a path length of 0.1 cm using a 3 μL Starna 20/C/Q,i/0.01 quartz cuvette (Starna Scientific, Hainault, Essex, UK). Circular dichroism spectra were recorded in the far UV region (190–250 nm) at a scan rate of 50 nm min−1 and an average of three scans were recorded on a Jasco J-815 circular dichroism spectropolarimeter (Jasco International Co., Tokyo, Japan) at 25 °C. Control samples (UltraPure DNase/RNase-free sterile distilled water) did not display any signal. High tension voltage was kept below a threshold value of 600 V.

2.6. Size Distribution and Zeta Potential Measurements

A Malvern Nano Zeta Sizer (Malvern Panalytical, Worcester, UK) was utilized to measure the average size and zeta potential of formulated peptide nanotubes using dynamic light scattering (DLS). Samples were measured using a low-volume disposable solvent-resistant polystyrene microcuvette for particle size measurements and a folded capillary zeta cell for zeta potential characterization (both Malvern Panalytical, Worcester, UK). For each size sample, an average of three measurements of 15 runs was made, agitating the sample between each run. For each zeta potential sample, three measurements were averaged each with up to 100 individual runs at pH 5.5 and 7.4. To measure the size and size distribution of individual nanoparticles, samples were analyzed using Malvern NanoSight NS300 (Malvern Panalytical, Worcester, UK) nanoparticle tracking analysis. Samples were diluted to 5 mL immediately before analysis and loaded into the chamber via a disposable syringe. For each sample three captures of 60 s were made, advancing the sample through the chamber using the syringe each time.

2.7. Tissue Culture Studies

Studies were performed to provide insight as to the potential inherent in anticancer activity of blank (no drug loaded) peptide nanotubes using tissue culture methods previously utilized by our group to establish cell cytotoxicity with murine subcutaneous fibroblast NCTC 929 cells.[22] Human glioblastoma cell line LN229 was cultured in DMEM supplemented with 5% v/v fetal calf serum. Cells were incubated at 37 °C, 5% CO2 and 5% fetal calf serum. Cells were incubated at 37 °C and 5% CO2 for 6 h. Following incubation, the cell culture medium was removed, and the adherent cells were carefully rinsed three times with PBS. CellTiter 96 AQueous One Solution Cell Proliferation Assay: Fresh cell culture medium containing 10 μL of MTS reagent was added and incubated for up to 120 min. At the experimental end point, absorbance of formazan at 490 nm was measured using a FLUOstar Omega plate reader (BMG LABTECH, Ortenberg, Germany). Percentage metabolic activity was calculated as outlined in Equation (1). Live/Dead Viability/Cytotoxicity Assay: PBS containing 4 μM ethidium homodimer-1 and 2 μM calcein AM was added to wells of a microtiter plate containing adhered cell lines treated with peptide nanotubes and incubated for 20 min. For imaging and counting, three randomly chosen areas and a total of 200 cells were counted. Cells were viewed on an EVOS FL microscope as outlined in Microscopy section above.

\[
\text{% Metabolic activity} = 100 - \left( \frac{[\text{Abs}_{490\text{nm, peptide}} - \text{Abs}_{490\text{nm, peptide}}]}{[\text{Abs}_{490\text{nm, 70% ethanol}} - \text{Abs}_{490\text{nm, media}}]} \right) \times 100
\]

(1)

2.8. Drug Loading, Drug Release, and Mathematical Modeling

2.8.1. Drug Loading

Sodium fluorescein was spontaneously incorporated into the peptide nanotubes by allowing self-assembly to occur in a solution where sodium fluorescein (0.0078125 mg mL−1, 21.27 mM) was dissolved in UltraPure DNase/RNase-free sterile distilled water. This concentration was achieved by diluting an initial stock solution of 0.5 mg mL−1 sodium fluorescein. Following self-assembly at room temperature for 24 h, the drug loading was quantified by sampling the supernatant and measuring the fluorescence (494/521 nm excitation/emission wavelengths) compared to a free unloaded sodium fluorescein control.[42] Sodium fluorescein was chosen as a model drug so that location of drug encapsulation could be visually assessed using fluorescent microscopy. The drug loading (%) was calculated as

\[
\text{% Drug loading} = \frac{\text{Quantity of drug added-Free unloaded drug}}{\text{Quantity of drug added}} \times 100
\]

(2)

2.8.2. Drug Release

One milliliter of each 10 mg mL−1 peptide nanotube formulation loaded with sodium fluorescein (0.0078125 mg mL−1, 21.27 mM) was placed into a glass scintillation vial containing either 9 mL of pH 5.5 acetic acid buffer or pH 7.4 PBS buffer and release of sodium fluorescein was measured after using an orbital shaker (Grant SS40-D shaking bath, Grant Instruments Ltd, Cambridgeshire, UK) at 100 strokes per minute at 37 °C.[43] Several time points up to 5 d (0.25, 0.5, 0.75, 1, 3, 6, 24, 48, and 120 h) were studied and at each time point 400 μL of buffer solution was removed from each glass vial and replaced with fresh buffer to keep the experimental volume constant. Samples were quantified using fluorescence spectroscopy (494/521 nm excitation/emission wavelengths) alongside a calibration curve.
KinetDS 3.0 software (SourceForge Media, La Jolla, California, USA) was utilized to characterize the in vitro drug release kinetics of sodium fluorescein from peptide nanotubes and identify the mathematical model that best fits from a range that includes Michaelis-Menten, Hill equation, and first-, second-, and third-order kinetic models of release.[44,45] The $r^2$ regression coefficient was used to determine the most appropriate drug release model by comparison of experimental data to predicted models.

2.9. Statistical Analysis

Statistical analyses were performed using Microsoft Excel 2013 and GraphPad Prism 6. Standard deviations were obtained at each concentration of peptide tested based on nine replicates for quantitative cell cytotoxicity assays and mean values obtained. Cell cytotoxicity (MTS, Live/Dead) and average nanotube size (DLS) was compared across different peptide concentrations to the media only negative controls. Average nanotube size (DLS) was compared using a one-way ANOVA with Tukey’s multiple comparisons test to identify individual differences. Percentage metabolic activity (MTS) or cell viability (Live/Dead) at each concentration of peptide was compared to the media only negative controls. Average nanotube size (DLS) was compared across different peptide concentrations (2.5–10 mg mL$^{-1}$) and to each separate peptide nanotube structure. Release of model drug from each peptide nanotube was compared at pH 7.4 and pH 5.5 using independent Student’s t-tests. Parametric ANOVA and t-tests were employed as data were shown to be normally distributed using the Kolmogorov-Smirnov and F-test methods, respectively. In all cases a probability of $p \leq 0.05$ denoted significance.

3. Results and Discussion

3.1. Formulation of Dipptide Nanotubes

In order to drive homogenous self-assembly the peptide must first be fully dissolved, most commonly through the use of organic solvents. However, this method creates several significant barriers for the pharmaceutical development and clinical translation of this technology due mainly to their toxicity but also environmental concerns. As outlined in Table 1, we proposed an alternative formulation strategy whereby peptide is suspended in distilled water, dispersed by a short period of vortexing (30 s) and fully dissolved by heating at 65 °C for 30 min. Our observations of FF solubilization in water at these temperatures correlate with those of the Shelnutt group.[49] It was important to ensure this formulation step occurred at relatively mild conditions to improve ease of future manufacturing upscale within the pharmaceutical industry and ensure thermal stability of peptide motifs. The large-scale manufacture of several pharmaceutical formulations require the use of heating at temperatures exceeding 65 °C including tablets (drying granules), ointments, creams, suppositories, and the preparations of solutions where poorly soluble preservatives are employed. Industrial steam-jacketed vessels are capable of manufacturing pharmaceutical up to 20,000 L volume at full production scale batch with temperatures up to 170 °C commonly employed.[46] Peptide nanotubes have previously demonstrated thermal stability in aqueous solutions upon autoclaving at 121 °C, therefore the selected temperature of 65 °C was deemed appropriate.[40] Self-assembly was allowed to proceed at room temperature for 24 h with the process of cooling serving as a trigger for nanotube formation via hydrophobic effect. Acuña et al.[47] also investigated the self-assembly of diphenylalanine-based nanostructures in water and in electrolyte solutions and studied the effect of varying electrolyte concentration and pH. They formulated tubes in water as a control using similar conditions of temperature but an acidic pH and found that nanotubes formed but interestingly the presence of salts, such as sodium chloride and calcium chloride, could promote the formation of long nanotube agglomerates that may be an important consideration in future in vivo studies. They proposed that this was due to the presence of salts enabling salt bridges to form between C-termini and between N-termini mediated by cations and anions, respectively. The formation of these bridges favors both longitudinal and radial tube growth therefore resulting in agglomeration. Furthermore, the in vivo fate of supramolecular assemblies such as these will depend on the location (e.g., vein) and form of administration because of the potential dilution effects due to factors such as blood flow, osmotic strength, and shear stress.

3.2. Microscopy

Electron microscopy is the most common method employed to measure the dimensions and observe the architecture of formulated nanotubes.[48,49] Microscopy may be advantageous compared to DLS in that the aspect ratio along with diameter and length of the nanoparticles in question can be quantified visually. However, these techniques are not ideal for self-assembled systems that tend to form aggregates of increased particle size due to a rise in analyte concentration during the drying step of sample preparation. This has been previously demonstrated for silver nanoparticle suspensions dropped onto a hydrophobic glass surface. The nanoparticles will aggregate during evaporation of water from the droplet to avoid the hydrophobic environments of the air interface and that of the glass surface.[50] It has also been reported that FF nanotubes can assemble on a microscale by stacking of hexagonal nanotubes into bundles. This may be driven by increased concentration gradients and would likely be difficult to differentiate using microscopy techniques alone.[51] In our study, nanotubes are visible in formulations of 1 mg mL$^{-1}$ H$_2$N-FF-COOH (Figure 1d–f) and H$_2$N-FF-NH$_2$ (Figure 1g) peptides, however the high density of tubes within the captured image impedes accurate measurement of individual tube length. Tube diameter of 0.320–0.365 μm (Figure 1d,e) and a pore size of 0.118 μm (Figure 1f) was demonstrated for H$_2$N-FF-COOH, smaller than the 2.2 μm tube diameter previously observed for this peptide when HFP

(Figure S14, Supporting Information $r^2 = 0.9961$). The study was repeated to obtain three replicates.
is employed at 10 mg mL\(^{-1}\) concentrations\,[51]\) but close to the 50–300 nm diameter range originally observed by Reches and Gazit.\,[52]\) Physicochemical parameters, for example, concentration, temperature, and type of solvent used for preparation have previously been shown to be important in controlling the size of these structures.\,[53,54]\) Although polydispersity is evident and it is clear that larger microtubes are present in the dried samples, these structures are far too large to be quantified by either nanoparticle tracking analysis or DLS. It is likely microtubes are artifacts formed during the drying steps in sample preparation. Therefore SEM is not a reliable tool for size determination in aqueous environments but provides a useful means of establishing whether self-assembly into tubular architectures has occurred. DLS and nanoparticle tracking analysis were therefore employed as a truer determinant of native nanotube size distribution.

### 3.3. Circular Dichroism

Circular dichroism (CD) spectroscopy provides insight into the folding-state of a peptide in solution, peptide self-assembly, and its secondary structure.\,[55]\) The presence of peptide nanotubes can be confirmed by CD spectra (Figure 2a–c). H\(_2\)N-FF-COOH and H\(_2\)N-ff-COOH are stereoisomers or nonsuperimposable mirror images and their CD spectra reflect this as they are mirrored along the x-axis, with corresponding peaks and troughs likely due to alpha-helix structures as recently reported for L- and D- FF nanostructures using molecular modeling techniques.\,[56]\) H\(_2\)N-FF-NH\(_2\) demonstrating smaller peak sizes suggests that there may be less assembly present. There was no alteration of folding observed at different pH values (pH 5.5, 7.4). The consistent secondary structures observed despite varying the pH, suggests that strong ion-pairing interactions are not crucial to the self-assembly process. This is consistent with the reports from Reches et al.\,[58]\) peptide analogues based on FF continue to form well-ordered tubular structures despite the lack of an overall charge. Dipeptides lacking an aromatic ring in their side chains have been shown previously not to assemble into tubular type structures, therefore there is a strong indication that the assembly process for nanotubes is driven by aromatic stacking.\,[57]\) pH-triggered delivery of drugs has been previously utilized by changing the folding configurations of peptides at different pHs. McCarthy et al.\,[58]\) report increasing the \(\alpha\)-helicity content of an amphipathic peptide RALA at lower pH, enabling destabilization of model endosomes. In practice, this type of environmentally triggered change in peptide secondary structure can be advantageous for releasing a drug cargo once the nanoparticle has been taken into the intracellular environment and physiological pH has altered. Although this may not be the case for FF peptide nanotubes, many nanoparticles also take advantage of the leaky vasculature and impaired lymph drainage in neoplastic tissues, allowing passive targeting and preferential accumulation in these areas for drug delivery in cancer.\,[59]\)

### 3.4. Apparent Size Distribution and Zeta Potential Measurements

The average apparent size and apparent size distribution of peptide nanotubes were quantified using DLS (Figure 2d) and nanoparticle tracking analysis (Figures S5–S7, Supporting Information). DLS provided an average apparent size range within \(\approx 90–260\) nm for all three peptide formulations between concentrations of 2.5 and 10 mg mL\(^{-1}\). No significant difference was observed in average size upon increasing peptide concentration or changing the FF structure. An average size, rather than individual length and diameter measurements, is provided by both DLS and nanoparticle tracking analysis. This is a limitation of its use in nonspherical morphologies such as tubes. However in such instances, the diffusion coefficient of the nanoparticle
is measured using light scattering and converted to the size of an equivalent sphere. Although not ideal, it provides data on size relative to different formulation parameters and has been previously used in literature for accurately reporting nanotube size. In a study by Krause et al.,[60] several commercial nanotubes with defined diameters of \( \approx 10 \) nm are dispersed in aqueous solution and measured by DLS on a Malvern Zetasizer. The z-average reported is in the range of 150–225 nm for the different brands of nanotubes. This is a similar size range to that demonstrated for H2N-FF-COOH, H2N-ff-COOH, and H2N-FF-NH2. Assuming that carbon nanotubes and FF peptide nanotubes have a similar aspect ratio, it can be inferred that the diameters are also of a similar magnitude.

Nanoparticle tracking analysis combines laser light scattering microscopy with a charge-coupled device camera.[61] The result is the ability to visualize, track and calculate the size of individual particles, as well as providing approximate nanoparticle concentrations. It has been demonstrated that in polydisperse samples, the size determination and peak resolution by DLS suffers due to the presence of large particles. As self-assembled systems typically have a large size distribution, nanoparticle tracking analysis may be a more appropriate technique for size determination. Figures S5–S7, Supporting Information show the apparent size distributions of H2N-FF-COOH, H2N-ff-COOH, and H2N-FF-NH2. Assuming that carbon nanotubes and FF peptide nanotubes have a similar aspect ratio, it can be inferred that the diameters are also of a similar magnitude.

Research into the effects of chirality on peptide self-assembly is in its infancy, however important differences have been observed. Molecular modeling and computational research recently conducted by Bystrov et al.[56] observed differences in D and L forms of homochiral diphenylalanine peptide nanotubes. D-enantiomeric H2N-ff-COOH was shown to form nanotubes thicker in diameter but shorter in length than its L-counterpart (H2N-FF-COOH). The authors attributed this to deeper and denser condensation of H2N-ff-COOH units during the self-assembly process. Denser and stronger packing allowed adjacent D-nanotubes to adhere more strongly in the direction of greater width and thickness. Recently this has also been observed experimentally by Zelenovskiy et al.[63] using a combination of optical microscopy and SEM.[62] Chirality’s effect on self-assembly propensity has also been observed by the Marchesan for peptide hydrogels, albeit for tripeptides and heterochiral forms.

From the perspective of drug delivery, nanoparticle size is an important factor for the ability to deliver therapeutic payloads. As discussed in the Introduction, the optimum size range for nanoparticles in drug delivery applications is within the range of 50–100 nm for all concentrations studied (Figure S6, Supporting Information). H2N-FF-NH2 demonstrates a slightly higher distribution of size in the \( \approx 100 \) nm range (Figure S8, Supporting Information). A similar profile is observed for D-enantiomer H2N-ff-COOH but interestingly larger particles are formed at higher concentration (10 mg mL\(^{-1}\)) as it assumes its highest percentage frequency of particles in the \( \approx 250 \) nm range.
the 10–200 nm range. This range is considered optimum to achieve efficient cellular uptake and favorable retention times while avoiding the opsonization associated with smaller particle sizes. Size distributions displayed in Figure 2d and Figures S5–S7, Supporting Information indicate that FF peptide nanotubes fall within this size range with the exception of 10 mg mL\(^{-1}\) \(\text{H}_2\text{N-ff-COOH}\). Due to the limitations of the sizing techniques and the high aspect ratio of nanotubes, the actual diameters are likely much smaller and the lengths much larger. Other high aspect ratio drug delivery vehicles include carbon nanotubes that have been heavily investigated for their drug delivery potential. Reports of carbon nanotubes being able to penetrate a variety of cell types, even under endocytosis-inhibiting conditions, have proven that tubular-shaped nanoparticles are indeed capable of cellular entry.[64] However, the ability of nanotubes to escape cells after cellular uptake is important and warrants further investigation. Carbon nanotubes have been observed to cross cellular barriers to enter the nucleus and mitochondria.[65] with Jin et al.[66] demonstrating that DNA-coated single-walled nanotubes could undergo exocytosis after entering cells via endocytosis. Rate of exocytosis showed a close relationship to the length of the nanotubes with slower expulsion as nanotube length increased.[67]

The zeta potential of the peptide nanotubes are slightly positive \(+20–30 \text{ mV}\) (Figure 2d). Zeta potential can be used as an indicator of two characteristics in nanoparticle formulations: stability and cellular interactions. Charges of magnitude greater than \(-25\) or \(+25\) are considered to be highly stable while those with smaller charges will have a tendency to aggregate over time. Zeta potential is a factor in predicting cellular interaction of nanoparticles, as mammalian cells feature a charge of their own, which is typically negatively charged at physiological pH.[68] The attraction of opposite electrostatic charges dictates that a positively charged nanoparticle will have an increased level of interaction and therefore uptake within mammalian cells. At 2.5 and 5 mg mL\(^{-1}\) concentrations the zeta potential of peptides varies, with \(\text{H}_2\text{N-ff-COOH}\) displaying a reduced positive charge \((3 \text{ mV})\) but this increases to the \(+25–30 \text{ mV}\) range observed also for \(\text{H}_2\text{N-FF-COOH}\) and \(\text{H}_2\text{N-FF-NH}_2\) at the upper 10 mg mL\(^{-1}\) concentration.

There have been no previous zeta potential studies for peptide nanotubes, however self-assembled peptide nanovesicles within this zeta potential range have shown to be stable for up to 1 month when stored in a sealed vessel at room temperature, displaying minimal variation in zeta potential and particle size.[58] Cell penetrating peptide nanoparticles have also proven to be efficient carriers for gene therapy drugs that are otherwise vulnerable to enzymatic attack. Positively charged nanoparticles have the ability to form complexes with negatively charged nucleic acids in a simple process of electrostatic interaction. Gold nanocomposites formulated to have either a positive or neutral zeta potential have also been compared in terms of biodistribution postintravenous injection.[69] It was observed that a neutrally charged formulation had a high organ uptake 1 h post injection with up to 18% injected dose per gram (ID g\(^{-1}\)) of organ in the kidney, liver, and spleen, while the positively charged formulation had 10.9% ID g\(^{-1}\) in the kidney alone and less than 10% in remaining organs. This suggests that a positive zeta potential may be favorable for prolonging circulation time and reducing uptake by the reticuloendothelial system. Peptide nanotubes within the \(+25–30 \text{ mV}\) range (2.5–10 mg mL\(^{-1}\) \(\text{H}_2\text{N-FF-COOH}\) and \(\text{H}_2\text{N-FF-NH}_2\) 10 mg mL\(^{-1}\) \(\text{H}_2\text{N-ff-COOH}\)) may therefore have a favorable zeta potential for stability, cellular uptake, and drug delivery applications, however further stability, in vitro cell and in vivo pharmacokinetics studies are required to conclusively test such hypotheses.

3.5. Tissue Culture Studies

Previous work by our group using NCTC 929 subcutaneous cells, the gold standard for in vitro biocompatibility testing,[70] established \(\text{H}_2\text{N-FF-COOH}\) and \(\text{H}_2\text{N-ff-COOH}\) to be biocompatible up to the highest concentrations employed in this paper (10 mg mL\(^{-1}\)).[22] However \(\text{H}_2\text{N-FF-NH}_2\) demonstrated toxicity against NCTC 929 at concentrations of 2.5 mg mL\(^{-1}\) and above. A separate study by Silva et al.[51] showed \(\text{H}_2\text{N-FF-COOH}\) microtubules to be biocompatible at concentrations up to 5 mg mL\(^{-1}\) (highest concentration employed) with 3T3 mouse fibroblasts. The aim of this study was to establish whether this toxicity profile correlated to LN229 cells isolated from a human glioblastoma cancer cell line and if peptide nanotubes possess inherent anticancer efficacy (cytotoxicity) against a malignant cell type. From the MTS assay results displayed in Figure 3a, the upper concentration (10 mg mL\(^{-1}\)) for all peptide nanotubes demonstrated a reduced metabolic activity to below 20% with LN229 cells. As previously established with NCTC 929 cells,[22] significant toxicity was also observed for \(\text{H}_2\text{N-FF-NH}_2\) at concentrations of 2.5 mg mL\(^{-1}\) and above in LN229 cells (Figure 3b,e). For \(\text{H}_2\text{N-FF-COOH}\) and \(\text{H}_2\text{N-ff-COOH}\) nanotubes no significant toxicity is shown at 10 mg mL\(^{-1}\) against LN229 cells using Live/Dead staining (Figure 3b,d) with the majority (>90%) of cells shown to be living. This does not match the MTS data results and suggests that while the cells are not dead, their metabolism using MTS reagent has been affected, relative to the untreated control. The MTS assay cannot directly measure the number of viable cells in a culture but rather measures an integrated set of enzyme activities that are related to cell metabolism.[71] The advantage of such an assay is the ability to cheaply screen a high number of samples with a relatively simple procedure, however, there are some limitations including the generation of false-positive results where specific aspects of cellular metabolism are affected and the inability to differentiate between cell cycle inhibition and cellular death. For example, in cases of excessive cell density or where the media is spent there will be a reduction in mitochondrial function that could result in an underestimation of cell viability if MTS were employed alone.[72] It is therefore prudent to employ a range of different studies in order to develop a more accurate, overall toxicity profile. The combined cell toxicity data suggest that a similar cell toxicity profile exists for LN229 glioblastoma and NCTC 929 subcutaneous for individual \(\text{H}_2\text{N-FF-COOH}\), \(\text{H}_2\text{N-ff-COOH}\), and \(\text{H}_2\text{N-FF-NH}_2\) peptide nanotube concentrations. \(\text{H}_2\text{N-FF-NH}_2\) is likely too toxic to differentiate between tumor and healthy cell lines. \(\text{H}_2\text{N-FF-COOH}\) and \(\text{H}_2\text{N-ff-COOH}\) demonstrate sufficient cell compatibility to potentially deliver target drug to tumors by acting as inert drug delivery carriers for chemotherapeutic agents.
3.6. Drug Loading, Drug Release, and Mathematical Modeling

To test how loaded hydrophilic drugs may be released from peptide nanotubes, they were first loaded with sodium fluorescein, a small hydrophilic molecule commonly used with cell/tissue permeability and fluorescence imaging studies. Sodium fluorescein was selected due to its low molecular mass (376 g mol$^{-1}$) and size (5 Å) allowing efficient loading by hydrophobic peptide nanotubes. It should be easily characterized having been used previously for labeling and visualization of nanoparticle-based systems. Drug loading is carried out by dissolving the sodium fluorescein in distilled water before self-assembly. This was shown to be successfully employed with over 85% loading observed for all peptide nanotube formulations (Figure 4a).

Fluorescence microscopy confirmed the presence and loading of fluorescent dye within the nanotube core (Figure S13, Supporting Information). PBS (pH 7.4) and acetic acid buffer (pH 5.5) were utilized as release media to model drug release at physiological pH and the acidic pH of the tumor microenvironment. The in vitro drug release profile of sodium fluorescein from all peptide nanotube formulations is displayed for the first 4 h in Figure 4b,c (full 120-h profiles: Figure S12, Supporting Information). A biphasic profile is observed showing an initial high burst release in the first 30 min, followed by a slower release rate for the remainder of the 5-d study. H$_2$N-FF-COOH demonstrated the highest rate of drug release, achieving 100% drug release at 3 h in pH 7.4 and at 24 h for pH 5.5. The rest of the formulations did not achieve complete release within the duration of the study with H$_2$N-FF-COOH and H$_2$N-ff-COOH achieving a maximum of 69.5% and 69.9% at pH 5.5, and 80.3% and 86.5% at pH 7.4, respectively. The observed release profiles are similar to those of poly(lactic acid), poly(glycolic acid) and their copolymer poly(lactide-co-glycolide) nanoparticles, loaded with a drug using noncovalent methods.

The release profile of these nanoparticles is usually biphasic, consisting of an initial rapid burst release followed by a period of sustained release. A reduced burst release and subsequent sustained release was observed for all peptides at pH 5.5 (Figure 4b,c), however this was shown to be nonsignificant when compared to release at pH 7.4. Sustained release of drug would have been preferential under acidic conditions allowing for possible reduced dosage frequency in cancer treatment. Acidosis is a hallmark feature of the tumor microenvironment where extracellular pH has been shown to range from 6.2 to 6.8 in solid tumors. Acidic pH can be exploited for targeted delivery of therapeutics with the benefit of less off-target activity in healthy cells/tissues. Nanomaterials stable at physiological pH can be formulated to facilitate drug delivery through protonation, acid labile bond cleavage, dissolvable pH-sensitive polymers, or other mechanisms when exposed to the acidity of the endosome or tumor microenvironment. Additionally, nanoparticles that enter cells via endosomes in the endocytosis pathway may experience a pH as low as 5. Release of drug at this pH may protect the therapeutic cargo by allowing endosomal escape and also offers a method of targeted treatment. An emerging method to target acidic environments
using peptides is low pH insertion peptides (pHLIPs), peptides capable of folding into helical structures that can insert across membranes due to an increase in hydrophobicity after being protonated. The charged amine terminus remains in the extracellular environment, while the carboxylic acid terminus of the peptide becomes deprotonated once it reaches the intracellular environment, resulting in an anchored transmembrane helix.\[79\] An initial rapid release of drugs may be undesirable as in most cases a sustained drug release, achieving a steady-state pharmacokinetics profile, is required ensuring adequate control of the disease state and reduction of potential dose-related side effects. For drugs with a narrow therapeutic window this is particularly important.\[80\] In some cases, for example acute infections, burst release may be intentional and can be triggered to release drug in a pulsatile manner in response to a specific environmental stimulus, such as temperature.\[81\]

Formulation strategies such as these may be key for the next generation of targeted and modified release therapeutics, however it may be difficult to employ them successfully within a dipeptide nanotube system in order to take advantage of both nanotube shape and pH-triggered delivery. Noncovalent methods of drug loading are not possible for dipeptide nanotubes as it is likely to negatively impact upon π–π stacking, hydrogen bonding, nanotube formation, and the self-assembly process.\[82\] Hydrogel-forming FF systems with a large aromatic terminus (Fmoc, naphthalene) are able to incorporate an extra amino acid, most commonly cysteine or lysine, capable of covalently linking a variety of low molecular weight drugs due to the minimal effect of drug on intermolecular π–π interactions between neighboring aromatic systems.\[83\] Higher molecular weight nanotube systems, for example cyclic-D,L octa peptides,\[20\] may be able to incorporate covalently attached drugs without detrimental effects to nanotube formation. Precise conjugation of drug to peptide may offer advantages in reducing rapid burst release of drug upon administration as observed when covalent drug attachment is employed for FF peptide hydrogelator systems.\[84\]

The mechanism of drug release can be investigated by fitting the data to various drug release models. Software such as KinetDS (SourceForge Media, La Jolla, CA, USA) is being employed more commonly to identify the mathematical model of drug release (Higuchi, Korsmeyer-Peppas, Weibull, Hixon-Crowell, Michaelis-Menten, Hill equation, and first-, second-, and third-order release) that best fits experimental data.\[44,45,85\] For H\(_2\)N-FF-COOH and H\(_2\)N-ff-COOH the release at both pH 5.5 and 7.4 is best modeled by the Weibull model, H\(_2\)N-FF-COOH has an \(r^2\) of 0.9851 at pH 5.5 and 0.9843 at pH 7.4. H\(_2\)N-ff-COOH has an \(r^2\) of 0.9864 at pH 5.5 and 0.9873 at pH 7.4 (Table S1, Supporting Information). The Weibull model describes many different types of drug release as it is an empirical and generalized form of the exponential function, and has been shown previously to be the most applicable model for drug release from nanoparticles.\[86,87\] H\(_2\)N-FF-NH\(_2\) shows a much greater rate of drug release than other formulations, with complete release being achieved within 120 h at both pH 7.4 and 5.5. For this formulation, release is also best modeled at pH
5.5 by Weibull with an $r^2$ of 0.896. Although existing published data on drug release from FF tubes are limited, Silva et al.\cite{5} have published a study investigating rhodamine B release from FF microtubes. The authors report that the constant release rate follows first-order kinetics, and 100% release is achieved by 3 h. It is not clear as to whether the authors tested other models of release, i.e., Weibull, although it is possible that changes in tube size or employing different model drugs, in this case rhodamine B, may alter mechanism of release.

Drug release from other nanotube structures has been previously reported primarily for carbon nanotubes. Drug release from single-walled carbon nanotubes loaded with anticancer drugs has been reported and modeled using various methods including zero-, first-, second-order, Higuchi, Riger-Peppas, and Weibull drug release.\cite{8} Under the conditions tested, the carbon nanotubes were reported to conform to second-order release with an $r^2$ of 0.999 and a proposed rate-limiting step of chemisorption. This type of drug release was also reported for silibinin-loaded carbon nanotubes.\cite{9} Although carbon and peptide nanotubes share similarities with regard to high aspect ratio, the difference in drug release kinetics is not surprising as carbon nanotubes show a high degree of stability, resulting in extended lifetimes within the host. Carbon nanotubes are engulfed by macrophages and remain distributed within the reticuloendothelial system.\cite{10} Zhang et al.\cite{11} showed that macrophages provided by RAW 264.7 and THP-1 cells were only capable of degrading 30% of a dose of carbon nanotubes after 9 d. The type of drug-nanocarrier bond will have a direct influence on the drug release profile. Both electrostatic and covalent bondings of drug cargo are environment dependent but covalent bonds are typically more stable and may require the action of a specific enzyme or water (hydrolysis) for cleavage.\cite{12,13}

Although the drug release profiles of FF nanotubes have been defined in vitro, future studies must focus on their activity in vivo and within specific disease states (e.g., cancer) or biological barriers (e.g., blood–brain barrier, cell membranes). In vivo pharmacokinetic profiling is essential for future clinical translation, with further data in this area allowing the FF peptide structure to be tailored to a particular profile of drug release (e.g., zero-order sustained release).

4. Conclusion

This work outlines the physiochemical properties of dipeptide nanotubes composed of FF formulated under mild conditions amenable to pharmaceutical manufacture and utilizing water-based solvents. This study outlines the possible use of such technology as pharmaceutical formulations primarily to deliver loaded hydrophilic drugs and avoid the use of toxic organic solvents. These results can also be used to expand the potential use of peptide nanotubes for drug delivery across biological barriers including the cell membrane to target intracellular organelles, the blood–brain barrier, bacterial cell membranes, the small intestine, and nasal, skin, and the oral mucosa. There is also huge potential to expand this technology to a variety of other applications for example cancers, antimicrobials, and vaccines. Cancers with poor prognosis, such as glioblastoma multiform, would particularly benefit from the study and application of new formulation strategies. Further studies are required to assess in vivo pharmacokinetic properties using specific drugs tailored to clinical applications and disease states.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

S.L.P., S.M.C. and S.P. contributed equally to this work. This work was supported by Royal Society (IE160588 and RG150171), Engineering & Physical Sciences Research Council (EP/S031561/1) research grants for GL and N. Ireland Department for the Economy Studentships for SLP and SMC. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

Keywords

drug delivery, formulation, nanomaterials, nanotubes, peptide
[82] T. Doane, C. Burda, Adv. Drug Delivery Rev. 2013, 65, 607.
[83] Y. Ling, Y. Gao, C. Shu, Y. Zhou, W. Zhong, B. Xu, RSC Adv. 2015, 5, 101475.
[84] J. Li, X. Li, Y. Kuang, Y. Gao, X. Du, J. Shi, B. Xu, Adv. Healthcare Mater. 2013, 2, 1586.
[85] C. Mircioiu, V. Voicu, V. Anuta, A. Tudose, C. Celia, D. Paolino, M. Fresta, R. Sandulovici, I. Mircioiu, Pharmaceutics 2019, 11, 140.
[86] M. Barzegar-Jalali, K. Adibkia, H. Valizadeh, M. R. Shadbad, A. Nokhodchi, Y. Omidi, G. Mohammad, S. H. Nezhadi, M. Hasan, J. Pharm. Pharm. Sci. 2008, 11, 167.
[87] J. Dredán, I. Antal, I. Rácz, Int. J. Pharm. 1996, 145, 61.
[88] X. Liu, D. Xu, C. Liao, Y. Fang, B. Guo, J. Drug Delivery Sci. Technol. 2018, 43, 461.
[89] J. M. Tan, G. Kirthivasan, P. Arulselvan, S. Fakurazi, J. Nanomater. 2014, 2014, 1.
[90] M. Yang, M. Zhang, Front. Mater. 2019, 6, 2834.
[91] M. Zhang, M. Yang, C. Bussy, S. Iijima, K. Kostarelos, M. Yudasaka, Nanoscale 2015, 7, 2834.
[92] A. Popat, B. P. Ross, J. Liu, S. Jambhrunkar, F. Kleitz, S. Z. Qiao, Angew. Chem., Int. Ed. 2012, 51, 12486.