OUTPUT 3	Development of innovative tech. for materials formulation (TRL4)
Project specific objective	1) Innovative pharmaceutical formulations and technologies
Output description	Development of both high throughput and high value manufacturing techniques (nanofibrous and nanocomposite matrix approaches) which allow commercially feasible production. Scale-up tests.
Project Output Target	2
Expected project specific result (s)	2 new innovative technologies which present new manufacturing capacity for dosage forms; advantages and/or enhancements in performance over conventional technologies and manufacturing methods will be demonstrated. It may include enhanced solubility and dissolution rate of poorly water soluble drugs compared to conventional dosage forms, enhanced uptake of drugs and therapeutics into biological issues, the ability to incorporate drugs and therapeutic agents into delivery systems with enhanced capacities and stability. The applicability to pharmaceutical applications will be demonstrated. Their potential to increase the competitiveness of SMEs via the use of GRAS status materials in new and innovative ways, with techniques accessible to typical pharmaceutical SMEs. No Patent are targeted.
Partner responsible	PP2 (University College London)
Other Partners involved	

Summary of the objectives, activities and achievements obtained during the project

There are 2 Deliverables composes the Output 3 as follow. These reflect the key **objectives** of output 3:

- D 1.3.1 Identification of novel GRAS status composite materials (delivered)
- D 1.3.2 Development of novel physical characterisation and modelling approaches (delivered)

Activities:

Overall, Output 3 relates to the development of new techniques for the development of drug delivery systems that are both effective and scalable (a key consideration for any system that may realistically be used in practice). Drug delivery is a field of pharmaceutical research that involves the generation of vehicles for therapeutic agents that deliver that molecule to the appropriate site in the body. While simple tablets and capsules may suffice for many drugs, there are many molecules which require much more specialist and sophisticated delivery systems and it is for these 'difficult' molecules, which arguably constitute over 50% of current low molecular weight drugs and all virtually all new biological drugs, that novel systems are required. We have focused on two types of problem drug, both of which are highly relevant to cutting edge therapy.

Two areas have been investigated:

Activity area 1: Design and Characterization of Cyclosporine A-Loaded Nanofibers for Enhanced Drug Dissolution

We have looked at how peptides may be given by the oral route. Molecules such as cyclosporine A, which is used to suppress the immune system following transplant and to treat autoimmune disease, has a very low water solubility and low bioavailability. If a means of increasing the dissolution rate of this molecule could be found, the clear implication is that both the speed and efficiency of absorption would be improved. We have developed a novel system which involves incorporating the molecule into a polymeric nanofiber, alongside the inclusion of surface active agents which form self-assembly structures (micelles) into which the drug may be incorporated. In effect we have combined three mechanisms of enhanced dissolution in one system (nanofibers with large surface area, solid dispersions of drugs in water soluble polymers, micellar systems). We showed a huge increase in dissolution for the molecule. Moreover, the method of preparation is simple and scalable using Generally Regarded As Safe (GRAS) materials, hence this is a viable new approach to enhancing dissolution and bioavailability.

The **achievements** can be categorised into:

- **Knowledge: Created/Increased skill and capacities.**

Activity area 2: Non-viral hybrid polysaccharide nanogel for gene delivery

We have collaborated with Prof Simon Waddington (UCL) who is an expert in gene therapy to develop a novel non-viral approach to delivery. Viral carriers are the norm for genetic material, but recently there have been significant worries regarding their immunogenicity, hence there is now a driver for non-viral systems, particularly as they are potentially less toxic and easier to reproducibly fabricate and scale. We have developed a novel nanogel formulation which we have shown is not only capable of delivering genetic material to the nucleus but also shows remarkable prolongation of gene expression (i.e. the therapeutic effect is prolonged). We are discussing a possible patent of this invention. Again the nanogel is composed of well recognized materials and the manufacturing method is scalable and industrially feasible.

The **achievements** can be categorised into:

- **Knowledge: Created/Increased skill and capacities.**
- **Socio-Economic: Patent application**
  We are discussing a possible patent of this invention. Again the nanogel is composed of well recognized materials and the manufacturing method is scalable and industrially feasible.

1) Description of the scientific and technological achievements

The activities can be summarised as follows, with a more detailed description of the outcomes provided subsequently.

Activity area 1: Design and Characterization of Cyclosporine A-Loaded Nanofibers for Enhanced Drug Dissolution

The study has indicated that the highly hydrophobic peptide, cyclosporine A (CyA), may be successfully formulated for enhanced dissolution using a combination strategy of firstly incorporating into polymeric micelles followed by further incorporation into nanofibers, using PVP as a water miscible polymeric matrix materials. The designed system presents several advancements including the use of materials with GRAS status, relatively high loading of CyA, improved water soluble formulation of hydrophobic peptide BCS IV drug Cyclosporine A as mixed micelle dispersion, with further advantages of high surface area by incorporation in PVP nanofiber which in turn has positive implications for the dosage requirements. A paper has been published in ACS Omega and a poster presentation was given on the topic: Electrospun Micelle-Loaded Nanofibers as a Novel Approach to Delivering a Highly Hydrophobic Peptide Drug at 10th APS Pharm Sci conference on 11th September 2019 meeting at University of Greenwich.

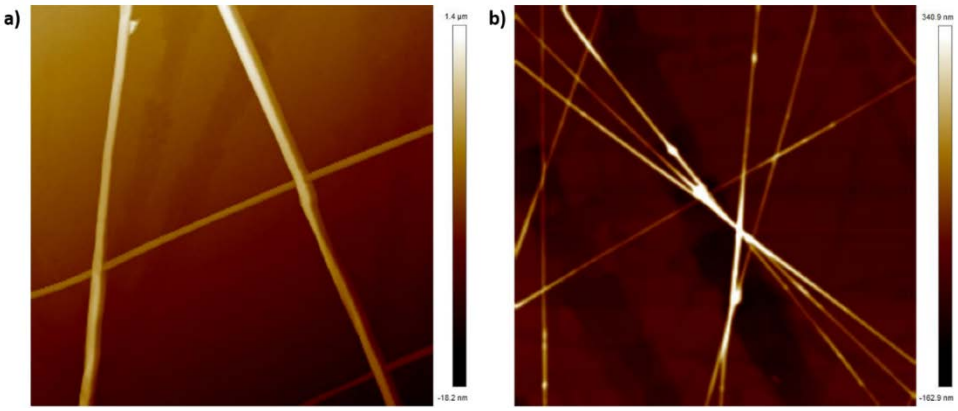

Figure 1: AFM topographic image showing a) PVP nanofibers and b) CyA/surfactant loaded nanofibers

Activity area 2: Non-viral hybrid polysaccharide nanogel for gene delivery

This work was performed as a proof of concept study, whereby a hybrid polysaccharide-PEI nanogel based nanoformulation has been designed and optimised for high molecular weight DNA loading. The nanogel transfection potential was studied both in vitro and in in vivo conditions. The developed non-viral nanogel technology presents a platform for an inexpensive manufacturing technology involving affordable low toxicity materials, simple and potentially scalable production and reliably small size. Our studies showed that nanogel systems produced a suitably small size with a high positive zeta potential which in turn augers well for stability. Further studies have demonstrated that our system shows good encapsulation, good transfection efficiency and high stability in both in vitro and in vivo conditions for extended periods of time in a new born mice model following intracranial injection. More specifically, our preliminary studies showed extended expression of genetic material in model HEK 293 cells after single dose nanogel treatment. Moreover the in vivo studies showed extended expression of luciferase expressing plasmid for more than 200 hours. The developed nanogel system offers the possibility for wide range of gene therapy based

applications. It can be further translated for cell based therapy and iPSC generations, and for gene specific targeting for particular disease conditions.

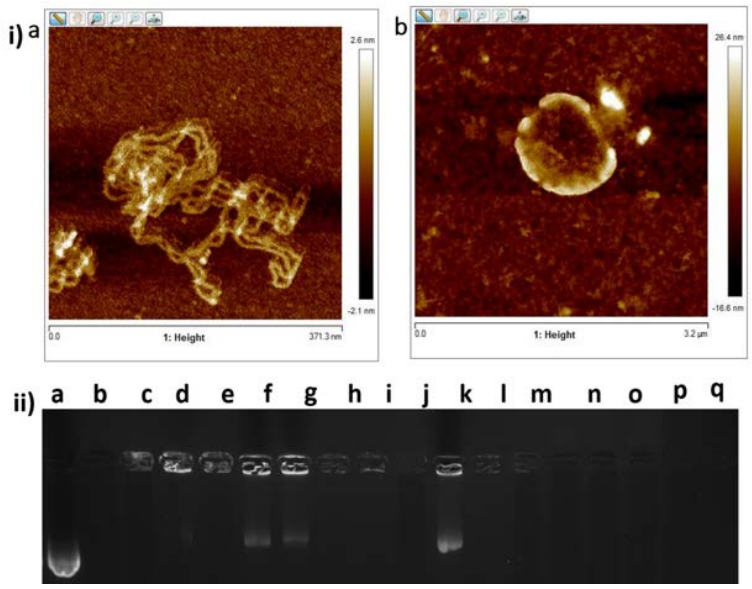

Figure 2: a,b) AFM topographic image showing structure of naked plasmid DNA and plasmid DNA loaded nanogel structure respectively, ii) Gel retardation assay of various N/P ratios of DNA/nanogel polyplexes (lane a, bare plasmid DNA; lane b, alone nanogel; lane, c-q, showing different N/P ratios of various polyplexes.

A patent application has been submitted to UCL Business (UCLB) UCLB with the title "A novel platform for nanogel-based non-viral gene delivery technology". The feedback from UCLB has been received and we are performing some further studies to strengthen the case. We are also planning to submit a research article from this work. At present further studies are being conducted to demonstrate the potential advantages of the system compared to available approaches as part of our building the case for the patent.

Therapeutic Acceleration Support (TAS) application was submitted to UCL on topic entitled "The development of novel nanogels as transfectants for non-viral gene editing: application to the treatment of Duchenne muscular dystrophy"; the application was not selected on this occasion, yet will form the basis for further applications as our data base continues to grow and support our approach.

Activity area 1: Design and Characterization of Cyclosporine A-Loaded Nanofibers for Enhanced Drug Dissolution

Despite widespread use as an immunosuppressant, the therapeutic efficacy of the undecapeptide cyclosporine A (CyA) is compromised when given by the oral route due to the innate hydrophobicity of the drug molecule, potentially leading to poor aqueous solubility and bioavailability. Cyclosporine A (CyA) is a cyclic undecapeptide, originally derived from the fungus *Tolypocladium inflatum*, and is used extensively as a therapeutic immunosuppressant due to its selective and reversible inhibition of T-lymphocytes, accompanied by low cytotoxicity[1]. More specifically, CyA is indicated for the treatment of conditions such as allograft rejection in transplantation, autoimmune diseases including psoriasis and rheumatoid arthritis, as well as dry eye disease. However, the drug is classified as Biopharmaceutics Classification System (BCS)

Class IV, with both low water solubility and membrane permeability, hence adsorption following oral administration is a major challenge due to both difficulties associated with transfer to the aqueous phase (a prerequisite for absorption) as well as limitations to subsequent transfer across the gastrointestinal wall. These absorption characteristics are associated with the physicochemical characteristics of the molecule, including high molecular weight (1,203 kDa), low water solubility (27.67 µg/mL at 25°C) coupled with high lipophilicity (log P = 2.92 at pH 7.4) and the very rigid cyclic structure of the molecule which provides a further architectural barrier to membrane permeation[2].

In this study we investigate the possibility of combining micellar and nanofiber delivery technologies as a means of enhancing, in the first instance, the dissolution rate of the drug into relevant aqueous media. This is a prerequisite to any enhancement of uptake due to the necessity of any drug being in a molecular disperse form prior to absorption. The ability of micellar systems to improve drug solubility via incorporation into those regions of the structure that are dielectrically compatible is well known. However, more recently the possibility of using nanofibrous systems as alternatives to matrix solid dispersions for the dissolution enhancement of poorly water soluble drugs has been outlined; not only may the fibers act as solid dispersions to present the drug to the aqueous media in a molecularly disperse form within a water-miscible base but the high surface area of the fibers further enhances the dissolution rate from the solid surface. Here we proposed to incorporate the CyA into micelles which are themselves incorporated into nanofibers, thereby providing a novel approach for dissolution enhancement combining micellar, nanofiber and solid dispersion technologies.

We examined the use of d-α-tocopheryl polyethylene glycol 1000 succinate (TPGS), an amphiphilic nonionic water-soluble derivative of vitamin E formed by conjugation of vitamin E succinate with polyethylene glycol (PEG). TPGS has generally regarded as safe (GRAS) status and is an FDA-approved pharmaceutical excipient; notably, it offers numerous advantageous features including solubilization of hydrophobic drugs, enhanced cellular uptake of the drug payload, and inhibition of P-glycoprotein to prevent drug efflux. However, it also has a relatively high critical micelle concentration (CMC) of circa 0.02% w/w, rendering TPGS micelles prone to dissociation on dilution, thereby potentially losing the solubilization advantage in the gastrointestinal tract. Consequently, generating mixed or composite structures with another co-polymeric material with a suitable CMC value represents a means of overcoming this difficulty. In order to achieve this, we explore the use of Pluronic F127 (henceforth referred to as F127) triblock copolymers, consisting of hydrophilic polyethylene oxide (PEO) and hydrophobic polypropylene oxide (PPO) segments arranged in a triblock structure (PEO100-PPO65-PEO100) with relatively extended PEO blocks. This material is commonly used as a low-CMC micellar carrier (0.0031%, w/w), while also having high biocompatibility and an acceptable safety profile and solubilization capacity. F127 has little effect on P-glycoprotein and does not significantly enhance membrane transport due to its relatively hydrophilic nature.

Our proposal was that by combining TPGS and F127 in mixed micelles we will generate a carrier system that is suitable for CyA from both a loading and a delivery perspective, while subsequent incorporation into nanofibers composed of the water-miscible polymer PVP will generate a highly hydrophilic system that not only offers a high surface area for dissolution but may also be further processed into a solid dosage form for oral delivery (this polymer being a common component of a range of oral dosage forms). In the first instance, the solubility of CyA in different Pluronic F127:TPGS ratios and concentrations was established, followed by characterization of the drug loaded micelles in terms of particle size, zeta potential, morphology, encapsulation and surface morphology. The micellar formulation was then loaded into PVP nanofibers and the product characterized in terms of physicochemical properties, *in vitro* release and

wettability studies. In this manner, the possibility of using micelle-forming nanofibers as potential carrier system for CyA may be effectively evaluated.

Overall, therefore, the aim of this study was to develop and characterize nanofibers based on the water-miscible polymer polyvinylpyrrolidone, incorporating CyA pre-loaded into polymeric surfactants so as to promote micelle formation on hydration; this approach therefore represents the novel combination of three dissolution enhancement methodologies, namely solid dispersion technology, micellar systems and nanofibers with enhanced surface area.

The preparation of the nanofibers was performed in two steps. Firstly, mixed micelles composed of the water-soluble vitamin E derivative d-α-tocopheryl polyethylene glycol 1000 succinate (TPGS) and the amphiphilic triblock polymer Pluronic®F127 (Poloxamer 407) were prepared. The micelles were characterized in terms of size, surface charge, drug loading and encapsulation efficiency using transmission electron microscopy (TEM), dynamic light scattering (DLS), Fourier-transform infrared spectroscopy (FTIR), high performance liquid chromatography (HPLC), and scanning electron and atomic force microscopy (SEM, and AFM) analysis.

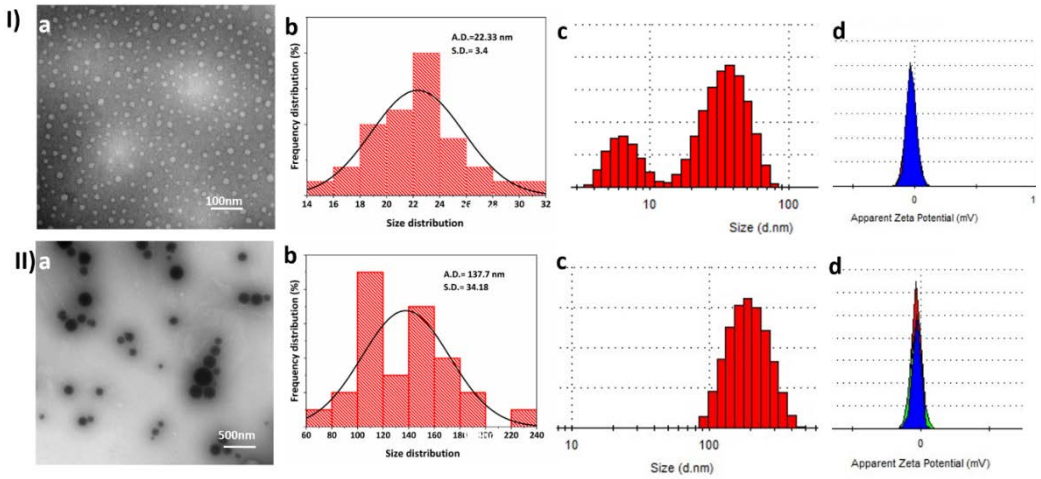

Figure 3. Size and associated data for (I) unloaded and (II) CyA loaded (F5) F127/TPGs (3:2) micellar systems. a) TEM micrographs; b) corresponding size distribution histograms (where A.D.= average diameter and S.D.= standard deviation); c) DLS based histogram with mean average hydrodynamic size of empty micelles 68 nm and CyA loaded micelles 180.0 nm d) corresponding zeta potentials with means of -3.26 and -8.7 mV respectively.

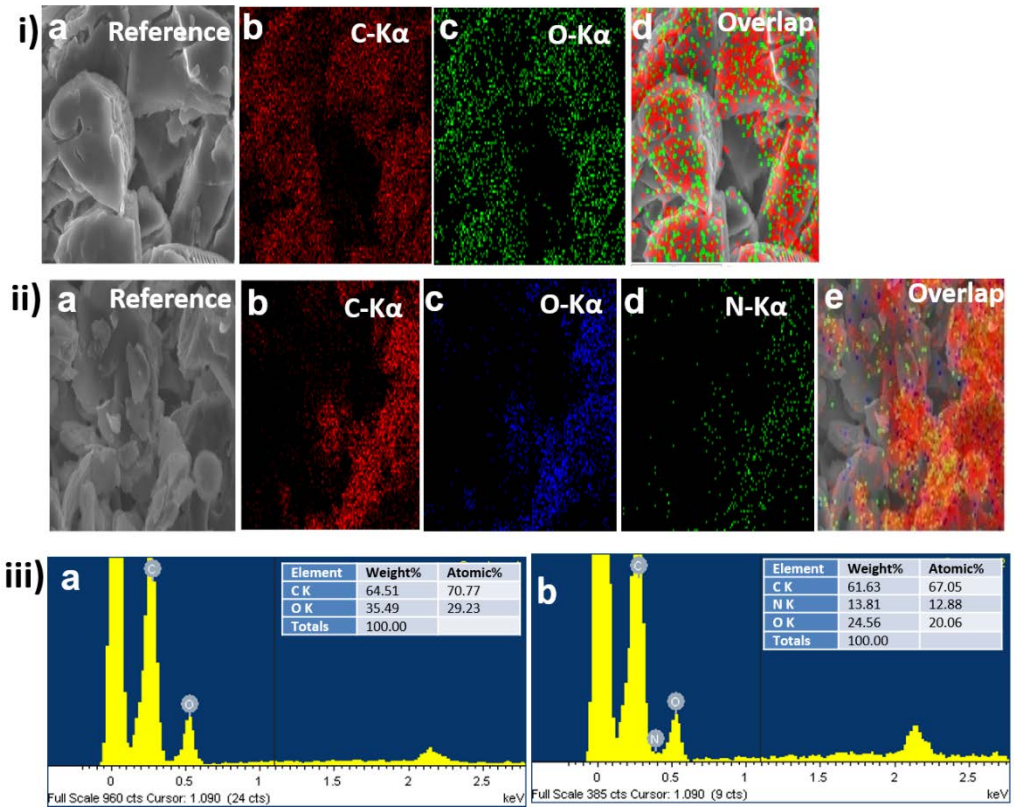

Figure 4. i) SEM-EDS elemental mapping analysis of unloaded micelles showing a) reference SEM image, b) Carbon (C), c) Oxygen (O), d) overlay of all element present, and ii) CyA loaded (F5) micelles including the reference SEM images a) reference SEM image, b) Carbon (C), c) Oxygen (O), d) Nitrogen (N) and e) overlay of all element present, iii) Point EDS analysis of a) the unloaded mixed micelles (no nitrogen peak detected) and b) CyA loaded mixed micelles (nitrogen peak detected as indicated). The inset table gives weight and atomic % information (iii a, b) respectively.

Nanofibers composed of poly(vinyl pyrrolidone) (PVP) and the drug loaded surfactant system were then prepared via electrospinning, with accompanying thermal, spectroscopic and surface topological analysis. Dissolution studies indicated an extremely rapid dissolution profile for the fibers compared to the drug alone, while wettability studies also indicated a marked decrease in contact angle compared to the drug alone.

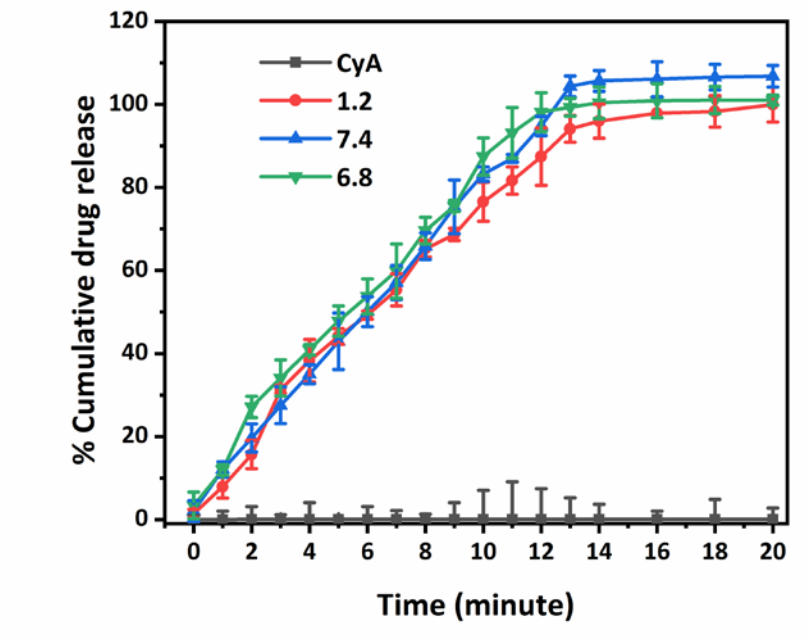

Figure 5. Dissolution profiles of CyA from CyA/micelle-loaded nanofibers at different pH values (1.2, 0.1N HCL, 6.8, PBS and 7.4, PBS) in comparison to the drug alone. The release for the nanofibrous systems after 14 min represent complete dissolution of the 200µg of CyA.

Overall, the new approach appears to offer a viable means of considerably improving the dissolution of the hydrophobic peptide CyA, with associated implications for improved oral bioavailability.

Activity area 2: Non-viral hybrid polysaccharide nanogel for gene delivery

This study relates to the generation of nanogels for the delivery of nucleic acid-based therapeutics using a new formulation strategy, with accompanying evidence for biological efficacy. More specifically, we describe a nanogel comprising a novel crosslinked polymer particle and an active agent (nucleic acid) contained within the nanogel in the form of a complex (polyplex), whereby the active agent is non-covalently associated with the nanogel via electrostatic interactions.

The currently available viral/non-viral systems present a somewhat complicated means of gene delivery, with limitations such as stability in serum conditions, delivery of only a specific size or molecular weight of gene due to the small viral capsid and poor size control in non-viral vectors[3-7].

Here we seek to develop the nanogel-based platform as an inexpensive manufacturing technology involving affordable biocompatible materials, simple and potentially scalable production and reliably small size. Our preliminary studies showed that our nanogel systems produced a suitably small size (measured by TEM and average hydrodynamic size) with a high positive zeta potential which in turn augers well for stability (Figure 6).

Our evidence for the efficacy of the nanogels lies in the following domains. Firstly, in terms of physico-chemical properties; our preliminary studies showed that our nanogel systems produced a suitably small size (measured by TEM and average hydrodynamic size) with a high positive zeta potential. We have also demonstrated a high encapsulation capacity, as evidenced by gel retardation assay data (Figure 2). The

formation of ionic complexes (polyplexes) between negatively charged plasmid DNA and the positively charged polymeric nanogel has been confirmed using agarose gel electrophoresis.

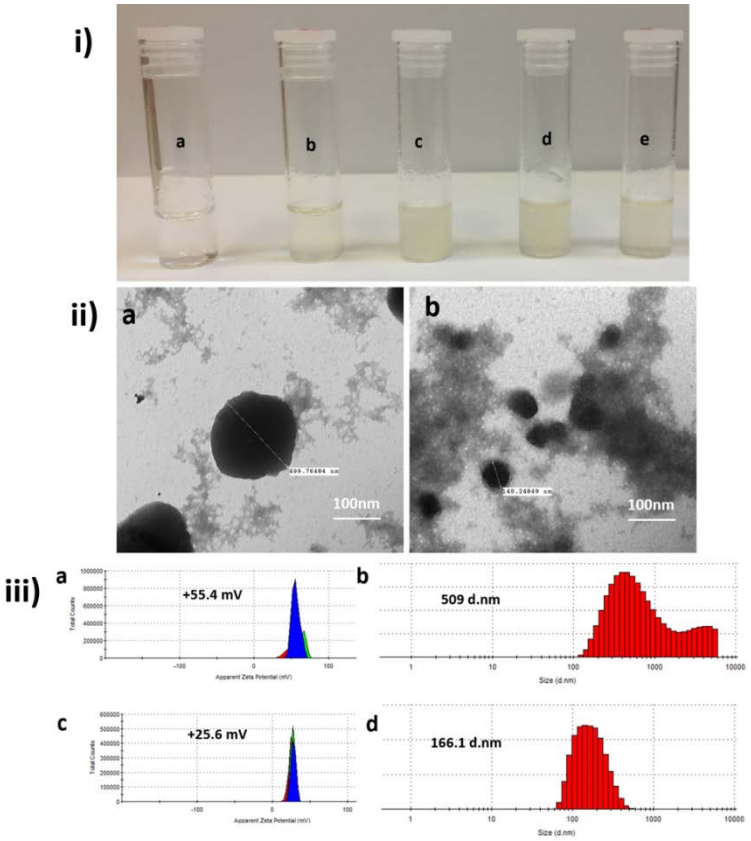

Figure 6: Appearance, size and zeta potential of hybrid nanogels

Secondly, we have demonstrated efficient and extended gene delivery both in vitro and in vivo. We have performed in vitro transfection studies, showing that HEK 293 cells expression GFP fluorescent protein over a period of 96 hours. The transfection capability of nanogel-based polyplexes was compared to the standard transfection agents including positive controls lipofectamine 2000 and PEI-25K. Qualitative analysis via fluorescence microscopy using the pGFP/luciferase plasmid expression system as the model genes showed the successful expression of GFP protein, confirming the cellular uptake, intracellular release and integrity of pDNA within the cells. Furthermore, the micrographs showed the expression level of GFP protein in cells treated with the nanogel system was comparable to cells treated with lipofectamine 2000 and PEI. Flow cytometry-based quantitative phenotyping (Figure 7) further confirmed the transfection efficacy of our hybrid nanogel system compared to standard commercial lipofectamine 2000. Our in vivo studies likewise showed that mice treated with nanogel-based polyplexes administered by intracranial (I.C.) injection showed extended expression of luciferase for more than 5 days, compared to 3 days for lipofectamine and PEI (Figure 8).

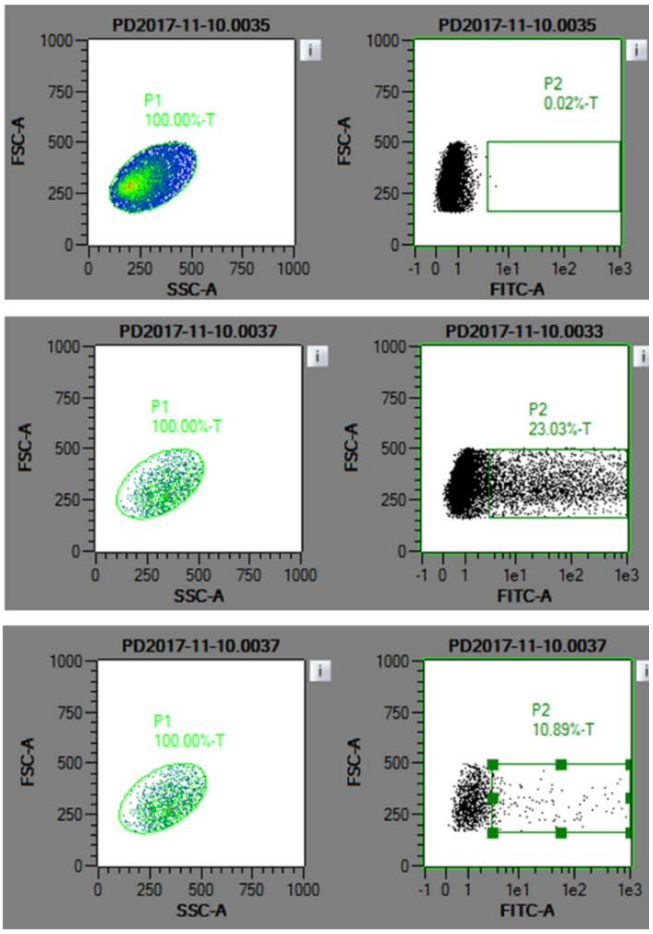

Figure 7: Quantitative uptake measured using flow cytometry showing a) untreated cells, b) lipofectamine-DNA complexes treated cells, and c) hybrid nanogel incorporated DNA) treated cells respectively

Thirdly, one of the key considerations for any gene delivery system is the cytotoxicity of the assembly. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) based colorimetric in vitro cytotoxicity assay was used to quantify the cytotoxic potential of several nanogel formulations using HEK 293 cell lines and compared to PEI. Significantly our nanogel-based system showed better cell survival than an equivalent concentration of PEI alone, showing favourable biocompatibility of the hybrid nanogel. transfection efficacy of our hybrid nanogel system compared to standard commercial lipofectamine 2000. Our in vivo studies likewise showed that mice treated with nanogel-based polyplexes administered by intracranial (I.C.) injection showed extended expression of luciferase for more than 5 days, compared to 3 days for lipofectamine and PEI.

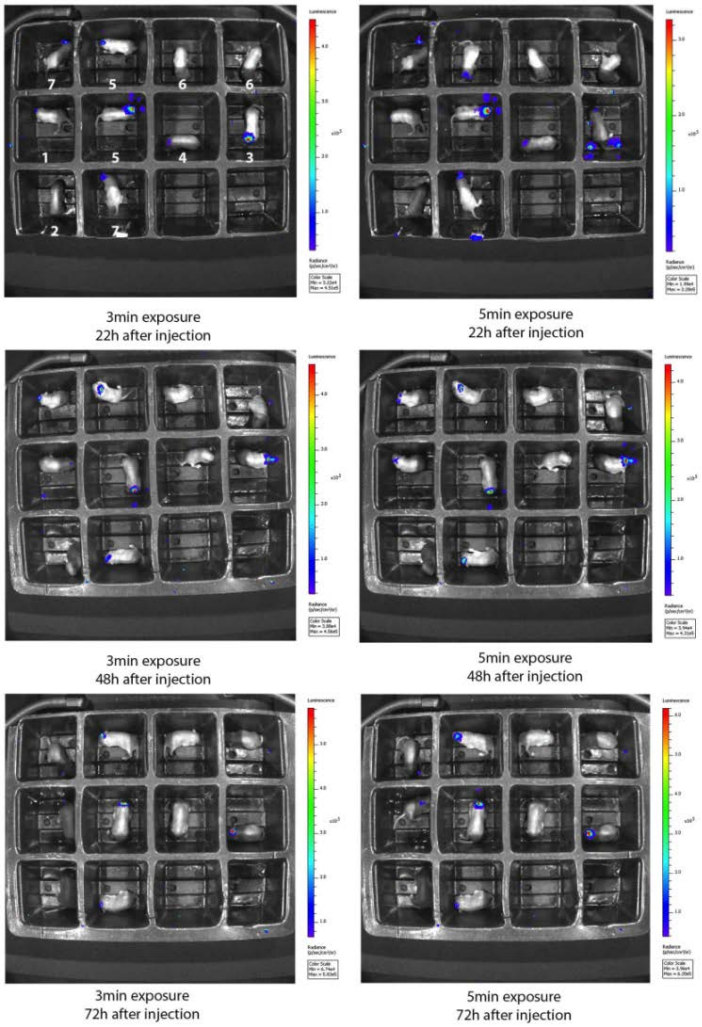

Figure 8: Intra-cranial injection into mouse model showed expression up to 72 hours using luciferase bioluminescence detection assay

Finally, one of the key considerations for any gene delivery system is the cytotoxicity of the assembly. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) based colorimetric in vitro cytotoxicity assay was used to quantify the cytotoxic potential of several nanogel formulations using HEK 293 cell lines and compared to PEI. Significantly our nanogel-based system showed better cell survival than an equivalent concentration of PEI alone, showing favourable biocompatibility of the hybrid nanogel.

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2) Description of the results obtained for the output in term of specific results category and specific result type

Specific results category And Specific result type	Description of the specific results
Knowledge - Created/Increased skill and capacities New technology #1: innovative new approach for the delivery of poorly water soluble molecules. This technology involves the incorporation of micelles into nanofibers, loaded with the poorly water soluble drug cyclosporine A.	1 Scientific paper published: Design and Characterization of Cyclosporine A-Loaded Nanofibers for Enhanced Drug Dissolution, P. Dubey, S. A. Barker, D. Q. M. Craig, ACS Omega 2020, 5, 2, 1003-1013. Results have been presented at: – IMODE Convention at Biofit conference (Marseille 2019) – 10th APS Pharm Sci conference on 11th September 2019 meeting at University of Greenwich.
Knowledge - Created/Increased skill and capacities New technology #2: a novel nanogel system for the delivery of genetic material. This technology involves the generation of novel nanogel systems that are able to incorporate and deliver genetic material for the enhanced expression of proteins in cells	Results have been presented at: – IMODE Convention at Biofit conference (Marseille 2019) Some data are protected due to patent possibility Three PhD projects generated from the nanogel work, hence this has generated a whole new paradigm of research
Socio-Economic -Patent applications Patent application for new technology #2 (see above)	Patent under discussion

List of documents enclosed as annex	
Images	
Reports and high impact publications	1 scientific paper published
Communications in European and/ or international events	1 poster presentation+ Duncan Craig intervention at BioFit
Patent prior art search & patent preparation	
Patent	
Official letters from company(ies)	