Flame-made calcium phosphate nanoparticles: a novel platform for antimicrobial drug delivery

V. Tsikourkitoudi, G.A. Sotiriou
Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, Stockholm, SE-171 77, Sweden

Statement of purpose: Nanomedicine has gained considerable attention recently and nanoparticle-based drug delivery systems are rapidly evolving to improve treatment for various diseases. Due to the novel properties that nanoparticulate carriers possess, compared to their bulk counterparts, such as high surface-to-volume ratio (large surface area/small size) and tunable chemistry, they have the potential to increase drug concentration at the diseased site, reduce toxicity and enhance therapeutic efficacy compared to free drugs. Most of the nanoparticle-mediated treatments approved for clinical use nowadays are using liposomes as drug carriers. Although various novel materials have been tested as drug delivery agents, there is still a long way from their demonstration in the laboratory until their large quantity production with reproducible properties that is necessary for their clinical use (Grodzinski P. ACS Nano 2019;13:7370-7376). In this regard, here we propose a novel drug delivery platform based on calcium phosphate (CaP) nanoparticles. We fabricate CaP nanoparticles by flame spray pyrolysis (FSP) that is a nanomanufacturing process famous for its scalability and reproducibility and assess their therapeutic potential against bacterial infections.

Methods: CaP nanoparticles were produced by FSP. The precursor solution, comprised of calcium acetate hydrate and tributyl phosphate dissolved in a mixture of 2-ethylhexanoic acid and propionic acid, was delivered to the flame through a capillary tube using a syringe pump and atomized in the FSP nozzle by O_2 gas at constant pressure (1.8 bar). The spray flame was ignited by a premixed supporting flame of methane/oxygen at flow rates of 1.5 L/min and 3.2 L/min, respectively. The synthesis of the particles was controlled by varying the precursor feed flow rate and the O_2 dispersion gas flow rate ratio. The particles were collected on a glass fibre filter with the aid of a vacuum pump.

We extensively characterized the as-synthesized CaP nanocarriers in terms of their physicochemical properties, i.e., specific surface area/size by nitrogen adsorption–desorption isotherms (Brunauer-Emmett-Teller, BET method), crystal phase and crystallinity by X-Ray Diffraction, and colloidal stability by Dynamic Light Scattering.

We further established an experimental protocol for loading biologics by physisorption on flame-made CaP nanoparticles. To establish the experimental protocol, we loaded bovine serum albumin (BSA) and bradykinin as model protein and peptide, respectively, and assessed the effect of their concentration and incubation time on the loading efficiency. We implemented the established protocol by loading antimicrobial peptides (LL-37 and a mannose receptor-derived one, MRC-1) on CaP and evaluated their stability against proteolytic degradation and their antimicrobial performance upon loading *in vitro* and *in vivo*.

In order to assess the enzymatic degradation of LL-37 and the protective potential from the CaP nanocarriers, we performed a degradation assay utilizing Proteinase K (Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis,SDS-PAGE). To verify if LL-37 retains its antimicrobial function after being physisorbed onto CaP nanoparticles, we studied its antimicrobial activity against both Gram-positive (*Streptococcus pneumoniae*) and Gram-negative (*Escherichia coli*) pathogens by monitoring the bacterial growth under treatment with different concentrations of LL37 either free or loaded on CaP nanoparticles.

Finally, we explored the potential of the CaP nanoparticles for *in vivo* delivery. We first visualized the biodistribution of the MRC-1-loaded particles in mice by administering fluorescently labelled peptide-loaded particles and imaging using an IVIS Spectrum-CT Imaging system and verified that the peptide loaded particles reach the lungs. Then, MRC-1 peptide loaded CaP nanoparticles were administered intranasally to mice previously infected by *S. pneumoniae.*

Results: High loading values, reaching 350 mg/g and 600 mg/g of CaP for BSA and bradykinin, respectively, are obtained, whereas maximum loading for LL-37 (~800 mg/g) outperforms all loading values found in the literature both for inorganic and organic nanocarriers (Tsikourkitoudi V. Molecules 2020;25:1747). Moreover, physisorption of LL-37 on CaP protects the peptide from enzymatic degradation, and it does not affect its antimicrobial functionality against both *S. pneumoniae* and *E. coli*. Similar antimicrobial activity for free LL-37 and LL-37-loaded CaP is observed for *S. pneumoniae*, whereas for *E. coli*, LL-37-loaded CaP exhibit better antimicrobial performance than free peptide. The administration of MRC-1 peptide loaded CaP nanoparticles is shown to reduce development of pneumococcal disease *in vivo* enhancing mice survival (Subramanian K. EMBO Mol. Med. 2020;12:e12695).

Conclusions: In a nutshell, we present nanocarrier synthesis by FSP, an intrinsically scalable and reproducible nanoparticle synthesis method. Typical fractal-like structure of agglomerated/aggregated nanoparticles made by FSP promotes high drug loading efficiency and enables the protection of the drug from enzymatic degradation, enabling its safe delivery (Starsich F.H.L., Annu. Rev. Chem. Biomol. Eng. 2019;10:155-174). High loading values achieved along with the *in vitro* and *in vivo* protective effect of the nanoparticles facilitate clinical translation of flame-made CaP nanoparticles as drug carriers.

Acknowledgements: This work was supported by European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program (ERC Grant agreement n° 758705). Funding from Karolinska Institutet Board of Research, Swedish Research Council (2016-03471; 2016- 00228; 2016-01861; 2018-05798), Jeansson Foundations (JS2016-0029), Torsten Söderberg Foundation (M87/18), and the Swedish Foundation for Strategic Research (FFL18-0043) is kindly acknowledged.