Bioengineering protein nanocages for applied nanomedicine: From fast prototyping to *in vitro* and *in vivo* delivery

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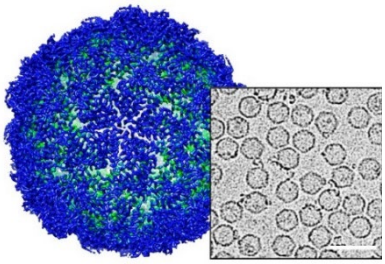

Encapsulin protein nanocages are a class of pseudo-organelles found inside some prokaryotes[1]. They self-assemble from identical protein subunits into hollow spherical nanoparticles (18-42nm) that exhibit solubility, biocompatibility *in vitro*, and, importantly, no toxicity *in vivo*. Encapsulin surfaces can be both genetically and chemically modified, permitting the precise attachment of functional moieties[2]. Uniquely, encapsulin subunits recognise and selectively assemble around cargo proteins. Excitingly, we exploited this functionality to load protein photosensitizers into encapsulins, resulting in nanocages that mediated *in vitro* photodynamic cancer therapy, thus showcasing the feasibility of encapsulins' as protein delivery vehicles[3]. **Our ongoing goal is to develop encapsulin nanocages into nanomedical tools, and here we present three areas of investigation we have focused upon recently: (1)** Establishing reliable prototyping methods to rapidly engineer nanocages; **(2)** Developing strategies to enhance the intracellular delivery of nanocages; and **(3)** Unravelling the *in vivo* behaviour, fate, and impact of nanocages.

METHODS

1) **Rapid prototyping**: Cell-free protein synthesis (CFPS) reactions were tested and optimised for the production/purification of encapsulin nanocages loaded with different cargo (e.g., enzymes).
2) **Intracellular delivery**: Cells were treated with nanocages and cell-penetrating peptide reagents, with nanocage uptake, endo/lysosomal escape, and intracellular delivery assessed by microscopic techniques.
3) ***In vivo* evaluations**: A preliminary *in vivo* biodistribution study was performed, in which nanocages were labelled with a near-infrared (NIR) dye and injected via tail-vein into mice. Post-injection, NIR fluorescence images were acquired over 24h

RESULTS AND DISCUSSION

1) **Rapid prototyping**: In microscale CFPS reactions (≥10ul), His-tagged encapsulins that differed in size/structure were successfully produced and then purified via Ni-NTA magnetic beads. Using this strategy, we prototyped the controlled encapsulation of different proteins inside nanocages, and also verified the integration of various unnatural amino acids into the nanocage which facilitated bioorthogonal conjugation chemistries.
2) **Intracellular delivery**: Primary mouse cortical neurons co-incubated with dye-labelled nanocages and cell-penetrating peptide reagents. Live cell confocal fluorescence microscopy revealed nanocages were present in the cytosol of neuronal bodies, indicating the free (non-endosomal) accumulation of nanocages inside the live neurons.
3) ***In vivo* evaluations**: In NIR images, free dye showed rapid elimination from the body, while dye-labelled encapsulins displayed slow liver deposition. At 48 h, less nanocages were observed in the liver, suggesting some excretion. Importantly, no negative behavioural changes were observed in mice, confirming that nanocages do not induce inflammatory shock and hypersensitivity reactions.

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