Commentary for *Nanomedicine (Lond)*

Functional recruitment for drug delivery through protein-based nanotechnologies

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The exploration and full development of nanostructured drug delivery systems (DDS) is expected to increase the chemical stability of the payload therapeutics upon systemic administration and to expand circulation time. By enlarging the size of the whole drug conjugate over ~5- 8 nm, but still within the submicron scale (up to ~500 nm), renal clearance and accumulation in lung are avoided while cell penetrability is preserved and often enhanced [1]. The need of nanoconjugates for drug delivery is especially well illustrated in the case of cancer therapies, in which the prescribed drugs are intrinsically cytotoxic.

In non-targeted antitumoral drug delivery loaded nanoparticles tend to escape the blood stream because of the leaky angiogenic endothelium and the aberrant lymphatic drainage in tumoral tissues. This enhanced permeability and retention effect (EPR) [2] is exploited as a way to promote the so-called ‘passive’ drug targeting in solid tumors. However, cancer chemotherapy has a low therapeutic index and promotes toxic effects that severely compromise patients’ life quality [3]. In this context, innovative cancer chemotherapies pursue cell-targeted drug delivery to enhance the effective drug concentration in tumor and metastatic foci while reducing drug doses, undesired side effects and fabrication costs. Targeted therapy could be achieved by functionalization of DDS, taking advantage of the overexpression of specific cell surface receptors in many cancer cells associated to the oncogenic process. In this context, the large surface/volume ratio of particulate nanomaterials offers opportunities for multiple chemical functionalization (Figure 1 A), aimed to recruit appropriate and sufficient agents to overcome the relevant barriers encountered before reaching the target

tissue [4], among which the most important is the cell membrane itself.

Cell penetrating peptides, especially those activated by features of cancer cells' environment, antibodies against cell-surface markers or tumor homing peptides (non-antibody ligands of the cell surface markers) are usually sufficient to reach good targeting and desired biodistribution of nanoconjugates. Endosomal escape and nuclear migration are additional functions that might be incorporated to DDS to enhance the therapeutic potential. Reporter agents such as quantum dots, fluorescent dyes or radioactive components confer additional values regarding theragnostic applications. The recruitment of these and additional functions in the drug complex is achieved upon chemical activation of the nanoparticle surface and its sequential functionalization [5] (Figure 1, i, ii, iii). The antitumoral drug is finally incorporated to the whole nanoconjugate (Figure 1, iv).

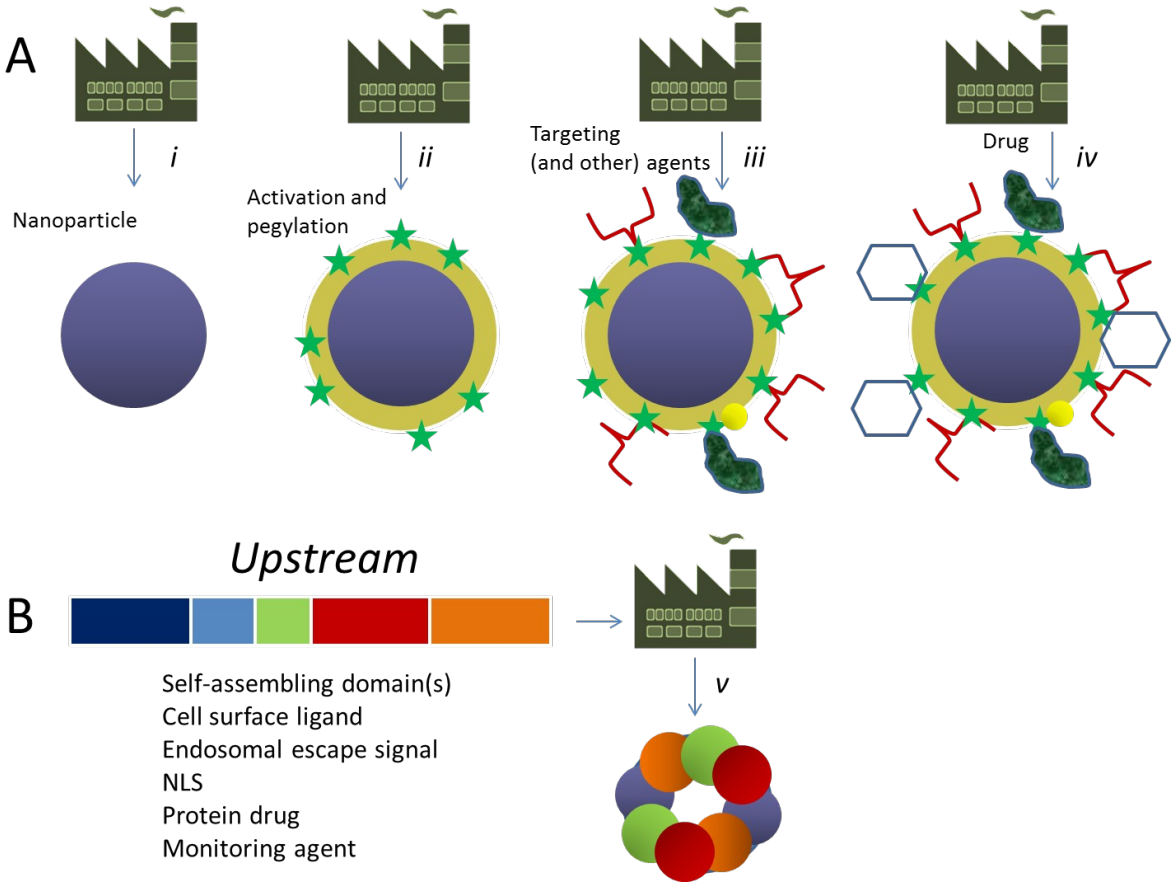

Figure 1. Conceptual design and fabrication pipeline of DDS. Step-based production of conventional cell targeted DDSs (**A**) versus one-step production of protein-based nanoparticles for protein drug delivery upon complex upstream genetic design (**B**). i. Nanoparticles (blue spheres) can be fabricated with different suitable materials including metals, dendrimers, natural and artificial polymers, carbon nanotubes, silica and others. ii. Before functionalization, chemical activation is performed by the inclusion of reactive groups (green stars) to further anchor the bioactive molecules. Pegylation (brown layer) often minimizes unspecific protein adsorption of the material in complex media and reduces the formation of protein corona [5], which is able to abort the activity of surface-attached cell targeting agents [6]. iii. Cell surface targeting is usually achieved by the addition of protein ligands (red symbols), namely antibodies, antibody fragments, non-antibody protein ligands or other specific bio-reactive molecules such as carbohydrates and aptamers. Other functional agents can be added here such as reporter molecules, including fluorescent chemicals, radiolabels and MRI labels (yellow spheres) that might be useful in preclinical studies for theragnosis. Importantly, the chemistry of conjugation should desirably control the positioning of the targeting ligands to maximize their reactivity with cell surface receptors [5]. Nuclear

localization signals (NLS, dark green entities) would ensure the active drug being delivered into the nucleus when it is required for therapeutic impact. iv. The drug itself (blue ring) is generally incorporated in a last step, although in some cases (eg liposomal constructs), it would be present already during nanoparticle formation for encapsulation. v. Since all the functions described in previous steps (cell surface recognition, penetration, endosomal escape, nuclear migration, fluorescence emission and cytotoxicity) can be reached by proteins, protein-based nanoparticles can be designed by conventional genetic engineering by recruiting functional domains from different origins (colored segments) at the upstream level. Self-assembling domains can be also added to a single chain polypeptide that upon biological production, results in fully functional nanoparticles in which the building blocks contain the drug themselves.

An alternative to the complex pipeline of heterogeneous DDS fabrication (Figure 1 A) is based on the protein nature of most of the functional agents used for cell targeting and intracellular trafficking, and also of many reporter fluorescent agents [7]. In addition, the drug itself might be a protein, since a wide set of cytotoxic peptides and proteins have been identified as active in antitumoral therapies, including pro-apoptotic factors, disintegrins and protease or protein kinase inhibitors [3]. Many of them are toxins from pathogenic microorganisms or components of animal or plant venoms [8-10]. Protein fusion technologies allow combining targeting agents with cytotoxic moieties in single chain polypeptides, in a pharmacological trend that is rapidly increasing [11]. A paradigm of such a drug (FDA-approved) is denileukin diftitox (ONTAK®), in which interleukin-2 (IL-2) is fused to a *Corynebacterium diphtheria* toxin and targeted to IL-2 receptors displayed on the surface of leukemia and lymphoma cell types. This modular concept allows a single-step production of the conjugate in recombinant microorganisms

(Figure 1 B), taking advantage of the versatility, scalability and cost-effectiveness of biofabrication [12]. Although the recombinant production of proteins excludes those composed by unnatural amino acids, the modular approach is offering intriguing roads in cancer nanomedicine by the generation of DDS based on single chain modular polypeptides.

Over the mere production of plain polypepides, the incorporation of self-assembling protein domains allow the generation of nano or micro-structured materials in which pre-defined structure and biological activities merge [13;14]. In this context, the fluorescent proteins IRFP and GFP have been recently engineered for a controlled self-assembly by either the addition of conveniently located cationic domains [15] or by the repositioning of inner barrel strands [16]. This approach generates protein-only recombinant nanoparticles with regulatable morphometric properties that if also containing cell-targeting peptides, achieve all the expectations of DDS regarding biodistribution and targeting [17]. The generation of multifunctional protein-only nanoparticles not only mimic the recognition patterns and *in vivo* behaviour of natural infectious viruses [18] but it also provides opportunities for an ‘a la carte’ functional recruitment of selected peptides and protein domains in tailored, chemically homogenous DDS.

The appropriate functional combination and refinement of self-assembling, cell-targeting and cytotoxicity in protein-only nanoparticles should result in a new category of antitumoral drugs suitable to be easily designed and produced by simple, one step recombinant technologies (Figure 1, v). Protein-only DDS are then an appealing alternative to conventional drug nanoconjugates not only regarding they intrinsically higher

biocompatibility but also in the context of avoiding sequential, biologically unfriendly and chemically complex functionalization processes.

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