Cellular Factories
Emerging Technologies for Fabrication of Nanomedicines?

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Abstract: The development of innovative nanomedicines requires the implementation of new biocompatible materials and their efficient assembly into defined nanostructures. Complex and costly synthesis of these materials can be coped with biological fabrication using microorganism factories, recombinant DNA and metabolic engineering. Modern bioprocess technologies may have the key for the implementation of tomorrow's nanomedicines. This paper specifically focuses on the current state of the art of nanopharmaceuticals and their future perspectives.

1 INTRODUCTION TO DRUG DELIVERY

The majority of clinically approved drugs are low molecular weight molecules (below 10^3 g/mol), which are often membrane permeable and generally spread throughout the whole body. As a consequence drugs reach healthy tissues as well as disease targets, which may result in unwanted side effects and/or rapid clearance and elimination. Non-specific biodistribution also results in a decreased therapeutic effect due to lowered accumulation at the target site. An effective approach to decrease side effects and enhance drug potency makes use of sophisticated delivery systems, several of which have crystalized in new approved therapies (Duncan 2003). Over the last years, multidisciplinar collaboration in biomedical research together with converging scientific technologies, such as nanotechnology and biotechnology have led to the development of modern nanomedicine (Duncan and Gaspar, 2011).

2 CURRENT STATE OF NANOMEDICINE

Nanomedicine is an overall term that has been defined by the European Science Foundation´s Forward Look Nanomedicine in the following manner: "Nanomedicine uses nano-sized tools for the diagnosis, prevention and treatment of disease and to gain increased understanding of the complex underlying pathophysiology of disease. The ultimate goal is improve quality-of-life".

Modern nanomedicines fit into three groups. The first group consists of first generation nanomedicines that have already entered routine clinical use and they include blockbuster products and certain products that are of such an age that they will soon begin to appear as generics. This group is mainly formed by technologies developed during the second half of the 20th century, such as liposomes (e.g: liposomal amphotericin B Ambisome (Lopez-Berestein, 1986) or liposomal doxorubicin Myocet (Mross et al., 2004)), polymer-protein conjugates (e.g.: styrene maleic anhydride-neocarcinostatin Zinostatin Stimaler (Maeda, 2001) or pegylated adenosine deaminase Adagen (Gaspar et al., 2009)) and polymeric drugs (e.g.: Glu-Ala-Tyr copolymer Copaxone (Johnson et al. 1995)).

The second group is made of an increasing

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number of nanomedicines in clinical development. It seems certain that a significant number of nanomedicines based on already approved delivery systems, such as liposomes and polymer-protein conjugates, encompassing new bioactives will continue to reach market approval. In addition, it is likely that other technologies, such as polymer- or antibody-drug conjugates (LoRusso et al., 2011), block co-polymer micelles (Hamaguchi et al., 2005) and/or nanoparticles (Wohlfart et al., 2011) will have their first regulatory approval and commercial success over the next decade, increasing the confidence of new technology approval.

Finally, the third group comprises innovative nanotechnologies, mostly still in pre-clinical or even proof-of-concept stages that may have the potential to enter clinical development. Many nanotechnologies are being continuously proposed for use as nanomedicines, such as carbon nanotubes (Wu et al., 2009), inorganic nanosized particles (Goel et al., 2009) or PRINT (particle replication in non-wetting templates) particles (Canelas et al., 2009). Significant progress in nanomedicine design together with the maturing of regulatory aspects experienced during the last decades are expected to fertilize the route towards a new paradigm to diagnosis and therapy.

Although it is difficult to predict the future in nanomedicine development, the lessons learned from first generation nanomedicines permits some speculation about preferred features and avoidable aspects of tomorrow's nanomedicines. Hence, it is important to emphasize that well-defined materials must be used for future developments, since many current nanomaterials are inherently heterogeneous. In addition, nanomedicines should preferably arise from rational design rather than a *let's try* attitude. For safety reasons, nanomedicines should be biodegradable to known and non-toxic metabolites or alternatively be engineered for efficient elimination via renal and/or hepatobiliary routes in order to avoid lysosomal storage disorders (Garnett and Kallinteri, 2006). Another important challenge is that emerging nanomedicines must be technologically feasible for large-scale manufacturing and processing to translate in cost effective novel therapies. However, fabrication of nanomedicines via synthetic approaches tends to be costly and technically difficult due to the large number of processing and purification steps. The purpose of this paper is to ponder whether nanomedicines of the future might be synthesized by biological means (bioprocessing). Such biofabrication platforms would represent direct and cost-effective systems for the production of complex nanomedicines.

3 FUTURE OF NANOMEDICINE

Evolution has furnished biological processes with an enviable level of control and specificity, which translates into exquisitely controlled hierarchical architectures at the molecular and supramolecular scale. These elegant structures and precise functions of biomacromolecules have inspired and continue to inspire strategies for nanomedicine development. However, it is likely that the next revolution in nanomedicine research will be fuelled by convergence of molecular and cellular biology with genomics, engineering and physical sciences to biofabricate nanomedicines (Sharp et al., 2011) rather than by construction of (bio)inspired macromolecular synthetic mimics or biological-synthetic hybrid structures (Pasparakis et al., 2010). The possibility to use the cellular machinery to entirely biosynthesize nanomedicines, would open the way to the development of innovative nanomedicines from new biocompatible materials produced by cost-effective fabrication methods, in contrast to difficult entirely synthetic methods. The biological fabrication of materials, mostly carried out by microorganisms, has historically provided biomacromolecules with wide-spectrum biomedical applications, including drugs (Engels et al., 2008), polymers (Liu et al., 2011), proteins (Ferrer-Miralles et al., 2009) and nucleic acids. Although microorganisms might be simply seen as reaction vessels for bioproduction, development of genetic and metabolic engineering is likely to render efficient platforms capable of producing complex nanomedicines, such as polymer-drug conjugates, protein nanoparticles or other nanoscale entities. The tremendous therapeutic potential of such organized and functional materials in nanomedicine prompts serious consideration of further exploitation of cell factories and recombinant DNA technologies as powerful alternatives to chemical synthesis. For this purpose, heterologous biosynthesis in engineering- and process-friendly hosts, such as Escherichia coli or Saccharomyces cerevisiae, of components and their subsequent assembly into finished functional nanomedicines, emerges as a promising technically feasible and cost-effective platform.

4 THE QUESTION IS: CAN THESE NEW BIOPROCESSED NANOMEDICINES BE MADE?

4.1 Polymer-Drug Conjugates

Microorganism-produced polymers are known interesting alternatives to synthetic polymers, as they are non-toxic, biocompatible and biodegradable materials. According to their chemical structure, biopolymers can be distinguished between polysaccharides, such as hyaluronic acid (Leonelli et al., 2008), polyamides, such as poly(γ-glutamic acid) (Choi et al., 2004), and polyesters, such as polyhydroxyalkanoates (Kim et al., 2009). Most of these polymers contain amenable sites for chemical modification – i.e. ligand conjugation or functionality introduction – that can render appropriate polymer tailoring for biomedical applications. Chemical conjugation of drugs to such polymers has been widely explored to produce polymer-drug conjugates that have been evaluated as potential therapies for cancer (Leonelli et al., 2008) and inflammatory (Yang et al., 2008) diseases. Some of these conjugates have had or are experiencing notable success, such as poly(glutamic acid)-paclitaxel conjugate (Opaxio), which is currently under phase III clinical evaluation (Galic et al., 2011). However, the production of such macromolecular constructs is often characterized by difficulties in their manufacture and processing. The reason for such costly development is the requirement of reproducible and specific procedures for chemical conjugation of the drug, followed by efficient purification of unreacted materials and by-products. Since a great number of drugs are obtained or can be obtained by microbial production, such as anti-cancer blockbusters doxorubicin and Colombo, 1999) and paclitaxel (Engels et al., 2008), it is not difficult to envisage that some of these polymer-drug conjugates could be obtained directly in bioprocess factories as a single final product. The development of a microorganism-based platforms capable of simultaneous production of both precursors – i.e. the drug and the polymer – followed by appropriate biotransformation mechanisms for successful conjugation of these precursors into organized nanostructures emerges as a promising system for the production of polymer-drug conjugates in a fast, technically feasible and cost effective manner (figure 1).

Notably, the main current limitation is the introduction of cellular mechanisms capable of conjugating the drug to the polymer chains inside modified microorganisms. Such systems would probably require the introduction of sets of enzymes capable of chemically linking the drug to the polymer in a site-specific, robust and reproducible manner. To the best of our knowledge, there are no references in the literature about known enzymes that mediate drug conjugation to polymers. However, research in this area is likely to identify enzymes capable of mediating specific polymer-drug conjugation. This is supported by the fact that a few enzyme-based approaches for peptide ligation have been already described. For example, sortase is an extensively studied transpeptidase found in the cell envelope of many Gram-positive bacteria that mediates transpeptidation by recognition of specific terminal aminoacid motifs at the C- and N-terminal of its substrates and ligands, respectively (Mao et al., 2004). Since sortase has shown transpeptidase activity in other non-amino acid primary amine-containing substrates, it is likely that the chemical structure of polymer and drug molecules may be engineered to make use of such enzyme-based coupling strategies (Ta et al., 2012). Another promising family of enzymes to be considered for enzymatic-based coupling of polymers and drugs are glycosyltransferases (Boltje et al., 2009); (Wagner and Pesnot, 2010).

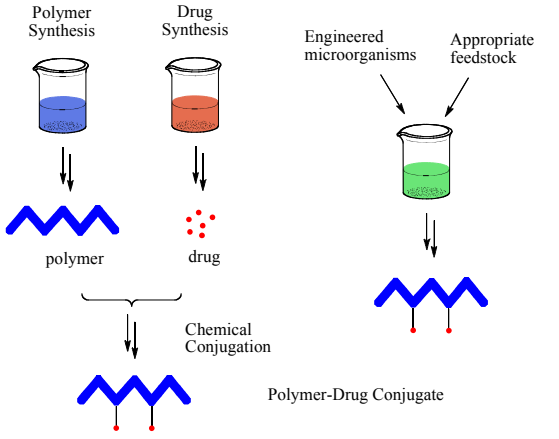

Figure 1: Synthesis of polymer-drug conjugates: synthetic *vs.* bioprocess approach.

4.2 Polymer-Protein Conjugates

Deficiency of specific proteins or non-functional versions of biologically relevant proteins may derive in diverse pathologies. Such disorders can be addressed clinically by administration of the missing protein to reach adequate physiological concentrations. However, in many cases therapeutic proteins

are very difficult to obtain from their natural sources and therefore bioprocess platforms using recombinant DNA technologies have been developed. Potent and relatively cost-effective production procedures can be achieved by cultivation of conveniently modified microbial cells, such as bacteria and yeast (Ferrer-Miralles et al., 2009).

Although there is an increasing number of approved recombinant proteins to be used as biopharmaceuticals, many of these therapeutic proteins face some limitations, which include short circulating half-life, immunogenicity, low solubility and proteolytic degradation (Duncan, 2003). A few strategies have been developed in order to improve their pharmacological properties for safer and more efficient use. Such strategies include changes in their amino acid sequence to reduce immunogenicity and proteolytic cleavage, conjugation to other proteins, such as albumin (Kurtzhals et al., 1995), or conjugation to natural or synthetic polymers (Roberts et al., 2012). The most efficient and versatile strategy so far consists on the chemical coupling of poly(ethylene glycol) (PEG). PEG conjugation can protect therapeutic proteins from premature clearance, proteolytic enzyme degradation and immunogenicity. In addition, PEGylation increases the apparent size of proteins, thus reducing renal filtration, which results in extended circulating half-life (Veronese and Pasut, 2005). Undoubtedly, PEGylation has made possible the clinical use of certain therapeutic proteins, whose administration compliance would otherwise be unfeasible. Despite clinical success, PEGylation of biologically active proteins may present drawbacks with respect to biopharmaceutical development and production, since additional *in vitro* processing and purification steps are required. Furthermore, the biological function of the therapeutic protein may be impaired, if chemical coupling takes place in the vicinity of its bioactive site. In addition, PEG is not biodegradable and may cause severe side effects, such as vacuolation of organs upon chronic administration. A wide arsenal of both synthetic and natural polymers, such as poly[N-(2-hydroxypropyl)-methacrylamide] (Johnson et al., 2012) and polyvinylpyrrolidone (Shibata et al., 2005) or polysialic acid (Pisal et al., 2010) or hyaluronic acid (Ferguson et al., 2010), have been explored as alternatives to PEG, however these systems do not avoid the need for additional processing and purification steps in order to obtain the final polymer-protein conjugates, and finally they have not shown superior performance than PEG.

Similarly as discussed earlier for polymer-drug conjugates, the development of bioprocess platforms capable of producing polymer-protein conjugates, either during protein synthesis (co-translational modification) or on finished proteins (post-translational modification), emerges as a promising alternative to polymer modification via chemical-based coupling strategies (figure 2). In this case, protein processing may also provide appropriate targeting signals to traffic the therapeutic protein to specific target sites.

A few alternative strategies to avoid synthetic post-modification strategies have been already proposed during the recent years, including glycosylation (Flintegaard et al., 2010) and genetic fusion of carrier proteins and polypetides (Cleland and Geething 2012). Modification of therapeutic proteins with glycans to prolong their *in vivo* half-life can be achieved by introducing mutations in their amino acid sequence in order to establish glycosylation sites. Glycosylation at these sites occurs via glycosyltransferase enzymes in protein processing events, either at the rough endoplasmic reticulum or the golgi apparatus. For success, the host platform requires to be suitably glycoengineered in order to correctly biosynthesize the therapeutic glycoprotein. Following this strategy, a first successful pharmaceutical product, Aranesp (glycoengineered erythropoietin), received market approval in 2001 and it is expected that others will follow. Although specific glycosylation might be useful for prolonging half-life, it may result in unwanted retargeting or increased immunogenicity. An emerging alternative to PEGylation and glycosylation of proteins is the post-translational enzymatic-conjugation of natural polysaccharides found in the human body, such as polysialic acid or hyaluronic acid. The hypothesis behind this strategy is that glycoengineered microorganisms could be used to produce PSA- or HyA-conjugated proteins in a single fermentation without the need for in vitro chemical modification.

Genetic fusion of either natural proteins, such as albumin (Sheffield et al., 2004), or unstructured polypeptide sequences of hydrophilic amino acids to either C,N-terminus or both termini of a recombinant protein provides a simple way to prolong plasma half-life and to diminish immunogenicity and proteolytic cleavage of biopharmaceuticals. Genetic fusion strategy allows biotechnological production of polymer-conjugated therapeutic proteins as one single product without the need of additional processing and purification steps. In addition, this system can be easily adjusted

to match the pharmacological needs by varying the polypeptide length and composition. Alternatively, targeting signals can be generated at C- or N-terminus to enable protein trafficking towards target tissues or cells. An interesting advantage of this technology is that in contrast to PEGylation genetic fusion of polypeptides renders a homogenous, monodisperse product with a defined chemical composition.

PASylation and XTEN technologies are two proprietary genetic fusion technologies that consist of disordered polypeptide sequences of Pro, Ala and Ser, and unstructured polypeptide containing Ala, Glu, Gly, Pro, Ser and Thr, respectively. It has been claimed that these technologies may reduce the cost of goods by up to 10-fold relative to PEGylation technologies.

A parallel alternative to genetic fusion is the polyglutamation and polyglycilation of therapeutic proteins, which consists of post-translational enzymatic-conjugation at the C-terminus of polymeric Glu and Gly, respectively (Janke et al., 2008).

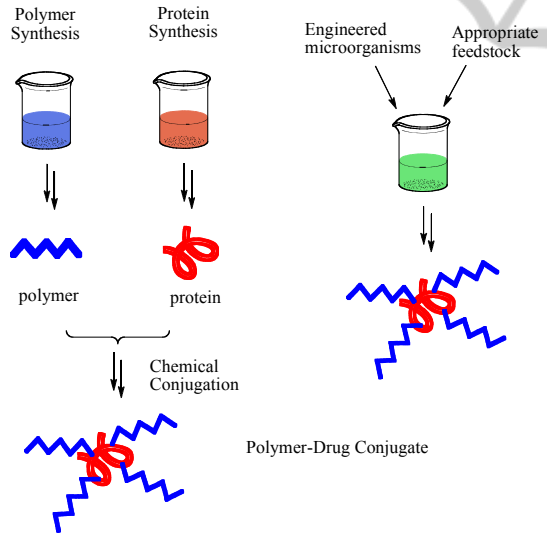

Figure 2: Synthesis of polymer-protein conjugates: synthetic *vs.* bioprocess approach.

4.3 Protein Cages and Nanoparticle Drug Encapsulation

Drug delivery systems based on drug encapsulation have been largely explored as potential therapeutic agents. In general, drug encapsulation enhances drug efficacy and reduces unwanted effects of free drug during trafficking to the target site. Lipid (mainly liposomes) and polymeric nanoparticles (i.e. PLA (Krause et al., 1985) or Abraxane (Zhao and Astruc, 2012)) have been under continuous development during the last decades and some products have already received market approval. These nanomedicines present some advantages, when compared to polymer-drug conjugation, including the protection of premature drug degradation and restricted interaction with the biological environment, preferential absorption into a selected tissue due to their nanoparticulate nature, bioavailability and retention time. Molecular organization, shape and size dispersion and drug encapsulation efficiency of these constructs is achieved by mechanical and chemical approaches. However, these constructs are obtained as rather heterogeneous mixtures. In addition, most of these particles require surface functionalization to enhance their pharmacological properties, mainly their half-life, and to present targeting motifs for specific and efficient trafficking to diseased tissues or cells.

Inspired by the monodisperse nature of viral particles and intracellular nanocompartments, it has been hypothesized that if properly adapted, these nanostructures could be turned into potent nanomedicine platforms. Additionally, due to evolution, viral particles posses specific targeting and cell-entry machinery, which are highly sought features in nanomedicine systems. Adaptation of viral particles as nanomedicine constructs via conventional chemical techniques would require casting of the genetic material and maintenance of the structural capsid for subsequent drug loading or conjugation and modification. However, one of the main limitations for using these constructs is the difficulty to have access to sufficient material of empty viral capsids, since viruses are obtained by culturing of host cells. Even if large amounts of viral capsids were available, chemical conjugation processes would be complex and expensive and would probably result in random attachment patterns and undesirable heterogeneity. For these reasons, a versatile bioprocess platform for the production of virus-like capsids or any other supramolecular structure suitable for accommodating drugs, small proteins or even nucleic acids in a cost-effective manner would be highly appealing (figure 3).

In this scenario, the development of bioprocess platforms capable of producing capsid proteins followed by macromolecular self-assembly could be exploited to engineer materials for encapsulation of active principles. Protein-based capsids are interesting vehicles for delivery applications, since they are biocompatible and their versatility of design would allow protein engineering to enhance vital

pharmacokinetic properties, such as prolonged half-life, enhanced proteolytic resistance and reduced immunogenicity.

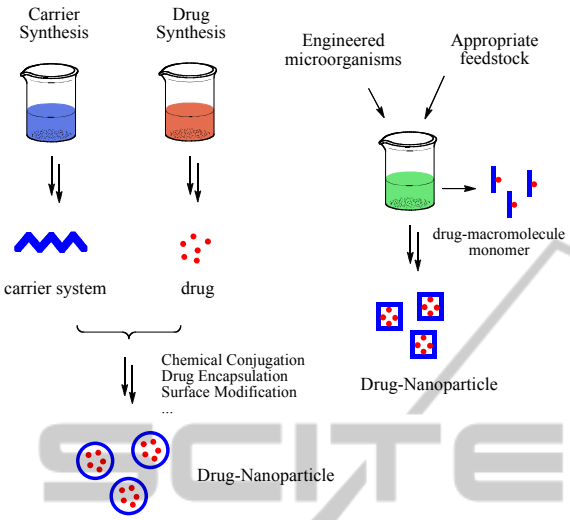

Figure 3: Synthesis of drug-nanoparticles: synthetic *vs.* bioprocess approach.

Mechanisms directing drug encapsulation within capsids, similar to the ones discussed for enzymatic polymer-drug or polymer-protein conjugation should be designed to direct drug conjugation to the inner surface of the capsid. A few early proof of concept works have demonstrated that it is feasible to encapsulate small enzymes in the interior of protein-based bacterial organelles both by specific enzymatic-based conjugation strategies at the inner side of the capsid proteins and by gene fusion of capsid and enzyme proteins (Fan et al., 2010). Upon macromolecular self-assembly, successful enzyme encapsulation inside the capsid was observed.

It is likely that the development of enzymatic-based conjugation strategies and gene fusion techniques to create specific docking sites in the interior of protein nanocages will not only allow the encapsulation of a wide range of therapeutic molecules, such as small drugs, therapeutic proteins, nucleic acids and imaging agents, but also the introduction of cell- or tissue-specific targeting motifs on the exterior and particle disassembly mechanisms for efficient release of the therapeutic load at the target site.

5 CONCLUDING REMARKS

Innovative nanoengineering together with increased knowledge arising from genomics, proteomics and metabolomics research brings exciting novel opportunities for nanomedicine development. There is a real chance to spur on modern nanomedicine development, as too many new nanomedicines still use old strategies and old drugs as the bioactive. Bioprocessing in the broadest conception of this term, including fermentation, biotransformation and downstream separation techniques in favour of new nanomedicine engineering may open the future to obtain more specific, defined and potent nanomedicine systems to improve patient therapy.

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