Targeting of injectable drug nanocrystals

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Abstract

"Nano" drug delivery carriers are established technologies for improving the therapeutic index of chemotherapeutic drugs and overcoming formulation challenges of poorly water-soluble compounds. Two important remaining challenges, however, are the need to formulate drugs on a case-by-case basis (due to the specific chemistry of each drug) and the difficulty associated with transporting large amounts of drug specifically to the site of the tumor (in part due to moderate to poor drug loadings). One of the most valuable "nano" opportunities in this field is to address these challenges by creating nano-carriers composed of the drug itself, in the form of so-called

nanocrystals. However, "nano" creates both opportunities and challenges for targeted drug delivery, which are critically discussed in both *in vitro* and *in vivo* settings in this contribution.

Keywords: Nanocrystal, Nanoparticle, Poorly-soluble drug, Targeted drug delivery, Chemotherapy, Layer-by-layer, Coating, Biodistribution, Dissolution, Shedding

Introduction

"Nano" drug delivery carriers are in many respects established technologies for improving the therapeutic index of chemotherapeutic drugs and overcoming critical formulation challenges of poorly water-soluble compounds. The latter are, for instance, difficult to administer intravenously (i.v.) due to their potential aggregation in the bloodstream, which can lead to embolism and accumulation in the lungs. A variety of anticancer drug nano-carriers based on *e.g.*, liposomes,[1] micelles,[2] polymeric nanoparticles,[3] etc., are in clinical trials, and some have reached the market. For example, liposomal doxorubicin (Doxil® or Myocet®) has now been used for more than 15 years in the treatment of myeloma, breast cancer, ovarian cancer, and AIDS-related Kaposi's sarcoma (see refs in [4]). In comparison to conventional drug formulations, nano-carriers can be advantageous because of the lesser use of solubilizing agents or co-solvents (excipients), which are often a source of side-effects such as hypersensitivity reactions (e.g., Cremophor EL® in the paclitaxel formulation Taxol®).[5] Furthermore, nano-carriers can reduce side-effects of the drug by targeting them specifically to sites of disease rather than to healthy tissues (*e.g.*, cardiotoxicity of free doxorubicin).[6] Two important remaining technological challenges of nano-carriers, however, are the need to formulate drugs on a case-by-case basis (*i.e.*, due to the specific chemistry of each drug) and the difficulty associated with transporting

large amounts of drug to the site of the tumor (*i.e.*, in part due to moderate to poor drug loadings).

One of the most valuable "nano" opportunities in this field is to address these challenges by creating nano-carriers composed of the drug itself.[7-11] This strategy reduces the use of "non-drug" material within the formulation compared to the other nano-carriers above, and, if successfully targeted to the site of disease, should deposit a significant amount of drug at this location. To this end, several drugs have been processed into colloidal dispersions known as "nanocrystals" (NCs),[12] and have been examined in cells, animal models, and in humans. In an excellent recent contribution, Gao *et al*.[13] describe the state-of-the-art of the *in vivo* performance of drug NCs. The NC platform is particularly attractive because production can be achieved by a variety of bottom–up and top–down approaches more-or-less irrespectively of the physical-chemical properties of the drug.[14, 15] Many techniques can lead to products with reduced particle size, including sophisticated ones such as laser fragmentation[16, 17] or supercritical fluids with enhanced mass transfer,[18, 19] but the most commonly used techniques in industry are high pressure homogenization, and wet milling.[20, 21] For example, wet milling can produce unimodal NCs with mean diameters in the ca. ~200 nm range with little batch-to-batch variability.[22] This process is suitable for many different classes of compounds and there currently exist a variety of oral NC formulations produced by wet milling on the market, including Rapamune® (sirolimus), Emend® (aprepitant), TriCor® (fenofibrate), and Megace® ES (megestrol acetate).[8, 23]

The opportunities that "nano" brings to targeted NC delivery. Due to their adjustable sub-~400 nm size, intravenously injected NCs can, in principle, extravasate from the blood through the leaky endothelium and accumulate in tumoral tissue *via* the enhanced permeation and retention effect.[24] In addition to this passive targeting phenomenon, the stabilizers used to mask

the high-energy drug surfaces created during the size-reduction process can be functionalized with targeting/internalizing ligands to promote active tumor accumulation or uptake, respectively. These stabilizers, for example polymers or surfactants, typically stabilize NCs by adsorbing to the surface of the NCs and providing steric (*e.g.*, poloxamers, cellulose derivatives)[25] and/or electrostatic (*e.g.*, sodium dodecyl sulfate)[26] barriers to aggregation.[12, 22, 27, 28] Steric stabilization is most efficient in a good solvent (for the stabilizer) and a minimum layer thickness is required,[29] while the comparable guidelines for electrostatic repulsion are that the absolute value of zeta potential should be at least 30 mV.[27] As only a small amount of stabilizing agent is typically required to mask NCs and prevent their aggregation, drug content of typically 50 to 90 wt% has been reported,[30, 31] which is dramatically higher than for other nano-carrier systems.

The challenges that "nano" brings to targeted NC delivery. NCs exhibit a characteristic non-linear increase of kinetic solubility upon miniaturization that is described by the Ostwald–Freundlich equation.[32] This phenomenon, which only becomes evident when particles reach the sub-micrometer size range, dramatically increases their rate of dissolution and is generally exploited for the "untargeted" administration of insoluble drugs, with little need for solubilizing agents or co-solvents.[13] However, enhanced dissolution complicates targeted NC delivery due to off-target drug delivery, insufficient circulation time for passive targeting, and potential shedding of the stabilizing agent/targeting agents used for active targeting. In addition, passive targeting via the enhanced permeation and retention effect is increasingly becoming a subject of debate. [33]

Overall, despite the fact that drug NCs have been studied and used clinically for nearly three decades, enhancing NC uptake through specific interactions *in vitro*, and targeting *in vivo* remain elusive objectives. This non-comprehensive review highlights the opportunities offered by NCs

as well as the important challenges that remain for achieving targeted delivery. For this purpose, selected studies on drug NC performance, irrespective of disease treated, in cell culture models and *in vivo* after parenteral administration are presented, and current and future avenues of research for enhancing their therapeutic potential are discussed. It should be noted that another advanced nanoparticulate system, namely Abraxane®, is also presented in this review. Abraxane® is an injectable paclitaxel formulation produced by high-pressure homogenization in the presence of human serum albumin and used for the treatment of metastatic breast cancer.[34] While not a (nano)crystalline material *per se*,[35] the clinical use of this product and its multiple physical–chemical similarities to other NCs supports its inclusion in this discussion, for comparative purposes.

In vitro

Cell-based assays are often used prior to *in vivo* experimentation to validate the performance of drug NCs in comparison to other formulations. In the case of anticancer drugs, the cytotoxicity of NCs compared to that of the free drug in solution or within other nano-carriers is used as a parameter for establishing activity. For drugs with limited cytotoxicity, uptake is either measured directly or *via* specific assays associated with their mechanism of action. Unfortunately, due to the specific conditions used in these assays, many studies involving drug NCs have yielded disparate results, which complicate generalizations and extrapolation of *in vitro* findings on NCs to the *in vivo* setting.

One fundamental, but sometimes forgotten, characteristic of cell culture assays is that they are performed within a closed system, of finite volume. As a consequence, the rate of drug dissolution from the NC, and its resulting consequence on therapeutic efficacy, can cease to depend on the dose administered if it is above the saturation solubility of the drug. In addition,

the relationship between incubation time and cytotoxicity will depend on the dissolution rate of the NC. For instance, when the contact time with cells is short, rapidly dissolving NCs of cytotoxic drugs should have a comparable effect on cell viability as the free drug in solution, and more slowly dissolving NCs should be less cytotoxic.[36] That is, when uptake is rapid compared to NC dissolution, structural or chemical parameters associated with the NC may play a role in performance. In support of this, Shegokar *et al*.[37] have shown that the *in vitro* uptake after 2 h of ca. 450-nm NCs formed of the antiretroviral drug nevirapine by macrophages was sensitive to the nature of the stabilizer, indicating that the NCs are still intact and have not shed their stabilizing coating within this time frame. The authors notably observed that the NCs coated with polyethylene glycol were less taken up by cells in comparison to NCs coated with dextran or albumin, in accordance with the stealth-like behavior previously reported for this polymer. NC endocytosis appears to be clathrin and caveolae mediated, as observed for 240-nm NCs of anticancer drug camptothecin with needle-like morphology. [38]

The situation changes when longer incubation times with NCs are used, given that dissolution may occur early in the overall incubation process. For example, 240-nm NCs of camptothecin showed similar cytotoxicity to that of the solution after 72 h of incubation. [38] Indeed, rapid dissolution has been observed in a number of reports. For instance, 125-nm tamoxifen NCs coated with three bi-layers of poly(dimethyldiallylamide ammonium chloride) and poly(styrene sulphonate) were ~50% solubilized within 2 h under sink conditions.[30] *In vitro*, Ben Zirar *et al*.[39] have evaluated the viability of both K562 and U937 cells after 48–72 h incubation with melarsoprol either as a free drug solution or as 300–600 nm poloxamer-stabilized NCs (Figure 1A). Under these incubation conditions, differences between the NC and the free drug, when statistically significant, were generally small. Vergara *et al*.[40] have assessed the cytotoxicity of

~150-nm paclitaxel NCs stabilized by electrostatic layer-by-layer (LbL) assembly of alginic acid and chitosan. Interestingly, the authors observed that cell viability ceased to decrease when the dose of paclitaxel was increased beyond ca. 5–10 $ng \cdot mL^{-1}$. At first glance, this result appeared to indicate that saturation (and thus prevention of NC dissolution) was occurring in the culture medium, despite the fact that these concentrations were well below the saturation solubility of paclitaxel in water (300 $ng \cdot mL^{-1}$).[41] However, only marginal differences were observed between the NC and freely soluble drug (Figure 1B), suggesting that dissolution had occurred well within this timeframe in cell culture medium. The authors attributed this phenomenon to the poor effectiveness of paclitaxel in OVCAR-3 cells due to the expression of the multidrug resistance transporter MDR1. This study points to the necessity of performing adequate control experiments with freely soluble drug. More recently, the uptake and intracellular trafficking of larger (ca. 300–900 nm) NCs of the antiretroviral drug ritonavir has been examined in macrophages.[42] Testing NCs of drugs with limited cytotoxicity allowed the authors to evaluate uptake at higher concentrations (100 μM). The tested NCs were stabilized with a mixture of poloxamer 188, 1,2-distearoyl-phosphatidyl ethanolamine-methyl-poly(ethylene glycol) (2 kDa), and 1,2-dioleoyl-3-trimethylammonium propane. The authors demonstrated by electron microscopy that these NCs loaded into macrophages and remained mostly intact (68%) 24 h post-uptake (Figure 2A). In addition, the NCs appeared to aggregate with time within the cells and possessed rougher edges. Sustained release of drug from the macrophages, which serve as drug NC reservoirs in this example, was observed for a variety of antiretroviral drug NC loaded macrophages for a prolonged period of time extending over a period of two weeks and longer.[43] In an extension of this work, the uptake of 21 different NCs (ca. 200–400 nm) of four antiretroviral drugs has been evaluated in macrophages under comparable conditions (100 μM).[44]

The authors observed that drug type, surfactant coating, and NC shape had substantive effects on NC uptake, release, and antiretroviral response. NCs with rounded and irregular edges showed diminished cell uptake, while rod-like NCs with smooth and regular edges were taken up more rapidly, and the loaded macrophages slowly released the drug in a period of days.

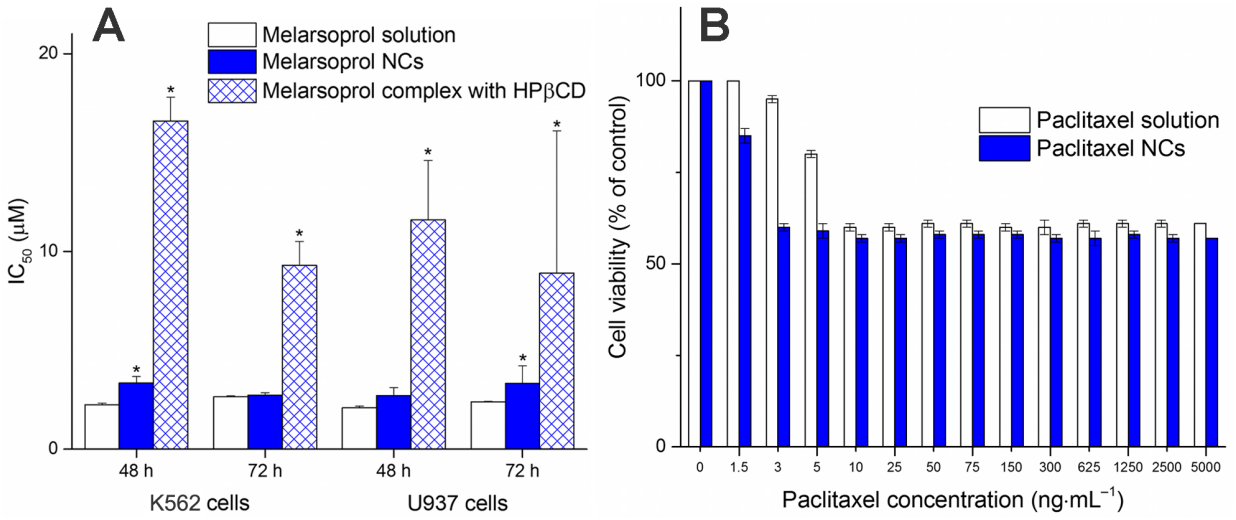

Figure 1. Drug solution-like behavior of NCs *in vitro*. (A) Viability of both K562 and U937 cells after 48–72 h incubation with melarsoprol either as a free drug solution, as 300–600 nm poloxamer-stabilized NCs, or as a drug–hydroxypropyl-β-cyclodextrin (HPβCD) complex (*: $p < 0.01$ *versus* free melarsoprol). Redrawn from Ben Zirar *et al.*[39], with permission from Elsevier (B) Cell viability of OCVAR-3 cells decreases as a function of paclitaxel concentration for both free paclitaxel, and paclitaxel NCs up to ca. 5–10 ng·mL^{-1} (24 h incubation time), after which it is unaffected. This phenomenon was attributed to the expression of the multidrug resistance transporter MDR1, rather than saturation of the medium with paclitaxel. Little differences are observed in comparison to the free drug. Redrawn from Vergara *et al.*[40], with permission from Elsevier.

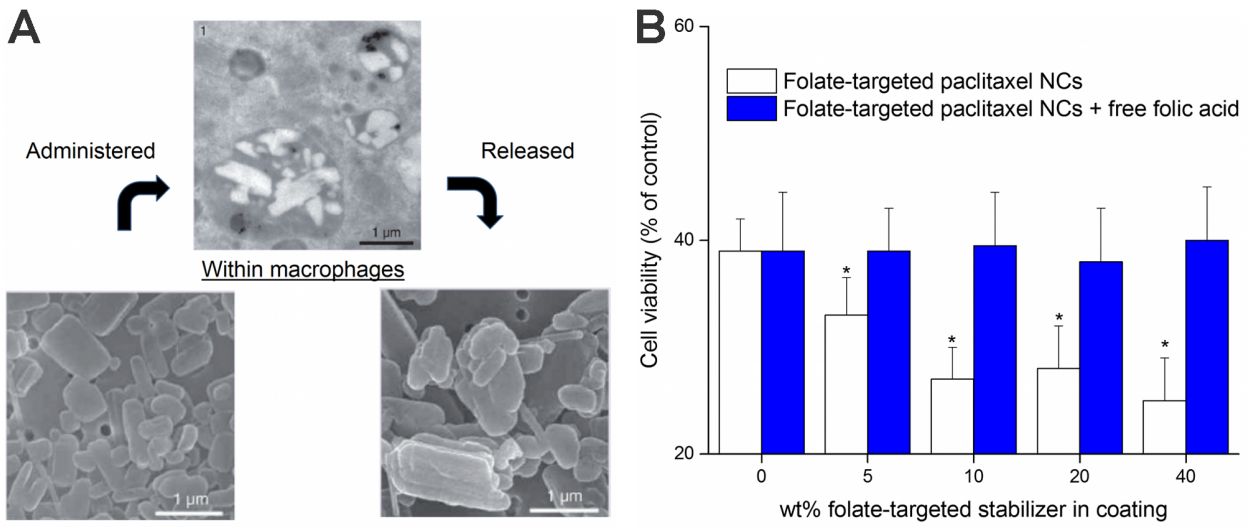

Figure 2. Particle-like behavior and enhanced NC uptake *in vitro*. (A) Electron micrographs of ritonavir NCs prior to macrophage uptake, within macrophages, and after release from macrophages into the surrounding medium (24 h after uptake). Adapted from Kadiu *et al.*[42], with permission from Future Medicine. (B) Paclitaxel NCs targeting the folate receptor are more cytotoxic than untargeted ones in a human folate-receptor-positive oral carcinoma cell line. The difference between the targeted and untargeted NCs disappeared when excess free folic acid was added to compete for the cell-surface receptor (*: $p < 0.01$ *versus* in presence of excess free folic acid). Redrawn from Liu *et al.*[36], with permission from John Wiley and Sons.

Despite the apparent ability to maintain, in certain cases, the integrity of NCs in the presence of cells over a certain period of time, to the extent of our knowledge, few attempts have been made to modify the surface of the NCs with targeting ligands to improve cellular uptake *in vitro*. Liu *et al.*[36] have shown that ca. 150-nm paclitaxel NCs coated with poloxamer 407 bearing 10% folic acid as targeting ligand were significantly more cytotoxic than the comparable non-targeted NCs at short incubation times (2 h; Figure 2B). This effect was abolished in a competition assay with free folic acid potentially indicating that drug uptake was associated with folate-mediated

receptor-mediated endocytosis. The cytotoxicity of the untargeted NCs was not affected by addition of folic acid. One caveat, however, is that cytotoxicity is an indirect measurement of drug uptake that cannot distinguish the folate-mediated uptake of free *versus* that of NC-associated paclitaxel. More recently, Bui *et al*.[45] have prepared fluorescent and biotinylated squalene–gemcitabine (prodrug) NCs and have observed increased cell uptake and improved anticancer efficiency in three cancer cell lines.

Overall, several cell culture studies support that the drug NCs can remain intact for a certain time and indeed behave like nanoparticles rather than freely dissolved drug. The dominant factors *in vitro* for maintaining NC integrity are size and concentration in the medium. Depending on the *in vivo* application foreseen, *in vitro* experiments might sometimes benefit from being performed under more dilute conditions. This would avoid saturating the medium with drug, which prevents NC dissolution. Dissolution profiles in the absence of cells and under sink conditions are indeed not always performed in the literature. Imaging of NCs within cells may also provide more insight into how the performance of NCs can be rationally altered.

In vivo

Quite often, NCs display pharmacokinetic profiles that are very similar to the drug solution when administered i.v.[46-49] This is generally a consequence of their rapid dissolution under *in vivo* sink conditions. In Mouton *et al*.'s[46] report on an early clinical study in humans, the authors compare 200–300 nm itraconazole NCs to an itraconazole–hydroxypropyl-β-cyclodextrin complex. The NCs exhibited a higher mean maximum plasma concentration at the end of infusion than those receiving the cyclodextrin formulation (Figure 3). The authors speculated that this difference may be explained by assuming that NCs were not yet dissolved, and were consequently confined in the circulatory system, and unavailable for diffusion and distribution to

the peripheral tissues. However, after this time point, the differences between the two formulations with regards to the other pharmacokinetic parameters was less pronounced or was not significant at all. In addition, the authors also mentioned that other (unpublished) data with several animal species showed that the drug NCs were specifically trapped in Kupffer cells in the liver and in the macrophages of the spleen and that pharmacokinetic changes were related to the size of the NCs (most pronounced for NCs ≥340 nm). The similar plasma concentration profiles and high drug concentrations early in the liver obtained for 200-nm NCs of an antitumor *p*-terphenyl derivative *versus* the drug solution could also be explained by fast dissolution and NC instability.[47] In a previous study this NC formulation exhibited complete dissolution within 2 h compared to less than 10% for the bulk drug.[50] Sharma *et al*.[48] have observed that the sub-150-nm NCs of the investigational anticancer compound SN 30191, stabilized with poloxamer 407 and poly(ethylene glycol)-15-hydroxystearate, were rapidly cleared from the blood of mice and accumulated in the kidney, liver, and heart. The authors postulated that drug accumulation in these tissues could be due to rapid dissolution of the NCs in the blood, which facilitated distribution in highly perfused tissues. Unfortunately, as the free soluble formulation of SN 30191 was four times less tolerated than the NCs, comparison between the two was not possible. Sigfridsson *et al*.[49] have compared 100–150 nm (amorphous drug) nanoparticles, 300–400 nm NCs, and the solution of the investigational antipsychotic drug AZ68. Both nanoparticles and NCs were stabilized with poly(vinyl pyrrolidone) and a combination of small-molecule surfactants. When administered i.v. to rats, no significant difference among the three formulations was observed in terms of their plasma profiles.

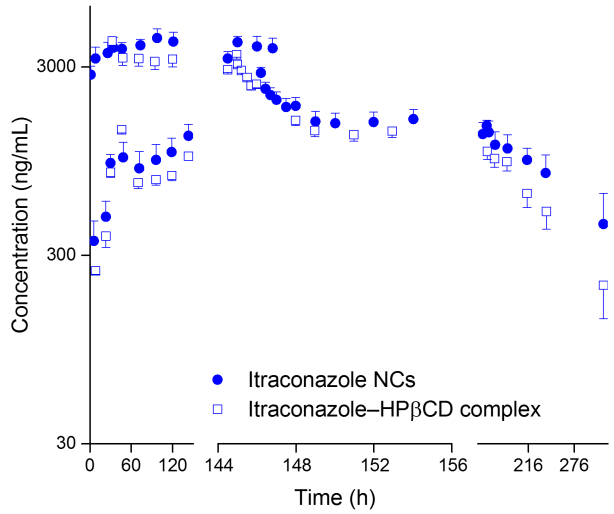

Figure 3. Drug solution-like behavior of NCs *in vivo*. Multiple-dose study comparing itraconazole NCs to an itraconazole–hydroxypropyl-β-cyclodextrin (HPβCD) complex administered i.v. to humans. Two hundred mg doses were given every 24 h except on days 1 and 2, when the dose was given every 12 h. Note that samples were collected just before and 1 h after each infusion for the first 5 days. Redrawn from Mouton *et al*.[46], with permission from American Society for Microbiology.

Differences between the NC and other formulations begin to manifest themselves when the particle size is large. Ganta *et al*.[51] have prepared 130–700 nm poloxamer 188-stabilized NCs of asulacrine and, when administered i.v. to mice, observed preferential accumulation in the liver compared to the free drug in solution. The authors rationalized this result to stem from uptake of the NCs by the mononuclear phagocyte system, which removed them from the systemic circulation. From the phagocytes, the drug was released over a period of a couple of hours, a timeframe that was consistent with the *in vitro* dissolution profiles of the NCs in 1% polysorbate 80 solution. This is in line with the observation of enhanced liver accumulation and retention of radioactive 450-nm nevirapine NCs in the liver compared to the drug in solution (Figure 4A).[37] Gao *et al*.[52] have investigated the effects of particle size on the pharmacokinetics and tissue

distribution of two oridonin NCs with markedly different size (ca. 100 and 900 nm) following i.v. injection in rabbits. *In vitro*, complete dissolution occurred within 10 min and 2 h, for the smaller and larger NCs, respectively. *In vivo*, the smaller NCs behaved similarly to the drug in solution, whereas the larger NCs accumulated to a greater extent in the liver, spleen, and lungs. Based on these findings, the authors suggested that the larger NCs were subjected to mononuclear phagocyte system uptake. Indeed, Rabinow *et al*.[53] have demonstrated, seven days post-injection, that 600-nm itraconazole NCs stabilized with poloxamer 188 were taken up intact by the spleen in rats by histological analysis (Figure 4B). Unfortunately, organ toxicity resulting from NC accumulation in the liver and spleen was so far not assessed. Nevertheless, such toxicity would be drug dependent and would have to be evaluated for each composition. In addition, continuous accumulation and organ toxicity, as observed for biopersistent nanoparticles, such as asbestos and carbon nanotubes, is unlikely since drug NCs dissolve with time and are eliminated metabolically and/or by renal excretion.[54, 55]

In contrast to the aforementioned i.v. injection, however, 200-nm rilpivirine NCs administered as a single i.m. or s.c. injection achieve stable sustained plasma concentration profiles detectable up to three months in dogs.[56] With respect to NC size, the 200-nm NCs displayed improved early release (higher C_{max}) in dogs, when compared with 400 or 800 nm particles. For instance, 40 × 150-nm paclitaxel NCs stabilized with D-α-tocopheryl poly(ethylene glycol) 1000 succinate (TPGS) exhibited greater antitumor efficacy than Taxol® at equivalent dose in a drug resistant NCI/ADR-RES xenograft mouse model.[57] Although it was hypothesized that the improved activity of the NCs could be attributed to the inhibition of efflux pump (permeability glycoprotein 1; P-gp) function by TPGS, as reported elsewhere,[58] the slow dissolution of the NCs observed *in vitro* (<20% in 24 h) might have contributed to a better drug distribution to the tumor

via the EPR effect. Unfortunately, in this study the pharmacokinetics and biodistribution of the drug were not assessed.[57] Zhang *et al*.[38] observed a greater antitumor efficacy for 240-nm uncoated NCs of camptothecin in an MCF-7 tumor xenograft mouse model compared to the salt solution of camptothecin in a mixture of propylene glycol and saline. They attributed this to the EPR effect and the higher resistance of NCs against hydrolysis. This was further supported by biodistribution data showing higher camptothecin deposition in the tumor and reduced drug hydrolysis. However, the pharmacokinetic profiles were difficult to interpret as the NCs displayed in rats a lower area under the plasma concentration *versus* time curve than the drug solution and a comparable mean residence time.[59] Based on previous data,[38] this may be indicative of retention of the uncoated needle shape NCs in the lungs but could also result from their aggregation and embolization in the lung capillaries. Hollis *et al*. developed 200-nm hybrid NCs consisting of partially radiolabeled paclitaxel and the fluorescent dye FPI-749.[33] Both NCs and paclitaxel solution, injected i.v. into HT-29 tumor xenograft bearing mice, accumulated less than 1% at the site of the tumor as determined by scintillation counting. Additionally, repeated injections of both formulations gave no significant difference in treatment efficacy at the defined endpoint.

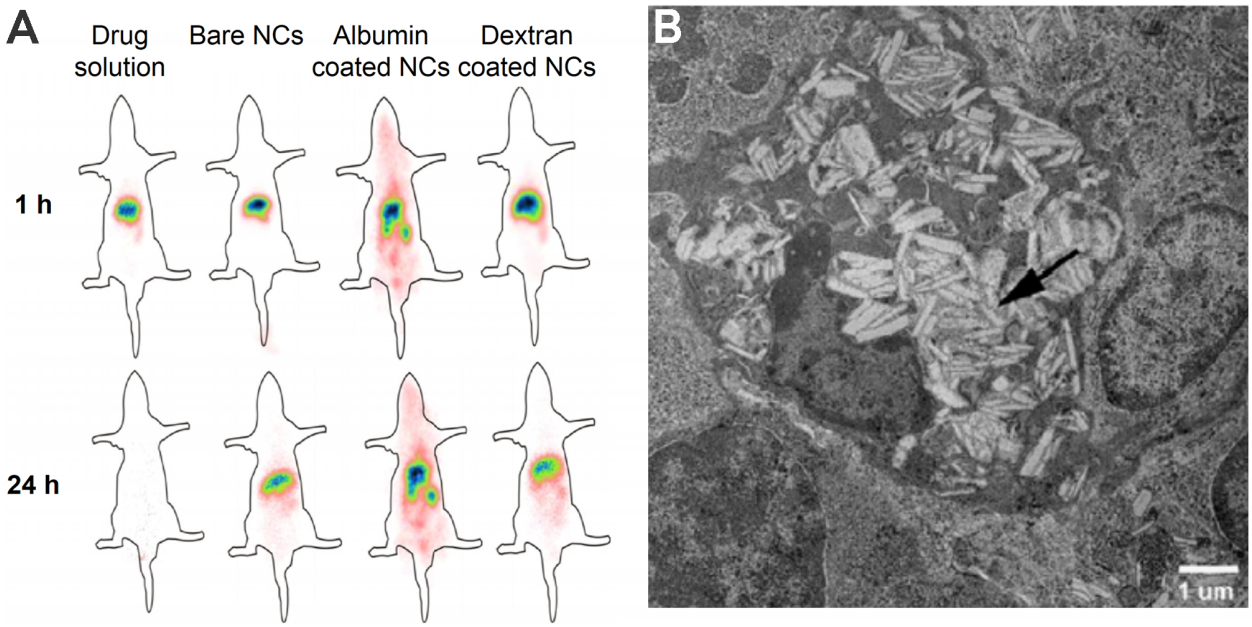

Figure 4. Particle-like behavior of NCs *in vivo*. (A) Gamma scintigrams depicting biodistribution of bare radiolabelled nevirapine 450-nm NCs and NCs surface-coated with albumin and dextran at 1 h and 24 h in rat compared to the drug in solution. Reproduced from Shegokar *et al.*[37], with permission from Elsevier. (B) Histological analysis of rat spleen by transmission electron microscopy shows the presence of itraconazole NCs within macrophages. Adapted from Rabinow *et al.*[53], with permission from Elsevier.

While not a NC *per se*, Karmali *et al.*[60] have modified Abraxane®, an amorphous paclitaxel nanoformulation stabilized with albumin (130 nm),[61] with tumor-targeting peptides and observed a change in the biodistribution 3 h post-administration *versus* the untargeted formulation. Indeed, at this time point, the targeted NC co-localized with its target while untargeted form did not. Unfortunately, targeted Abraxane® only showed a small effect in inhibiting MDA-MB-435 tumor growth in comparison to its unmodified form. This may attest to some dissolution prior to efficient targeting of the NC, and should be further investigated.

Stabilizer – to shed or not to shed

One important limitation in the design of therapeutic drug NCs is the desire not to chemically modify the NC itself with the stabilizing agent. As such, stabilizers cannot be covalently anchored onto the NC surface, and non-specific interactions must thus be exploited for adsorption. Independently of the dissolution of the NC that leads to desorption of the stabilizer (*vide infra*), high dilution conditions encountered either *in vitro* or *in vivo* will inevitably lead to loss of the stabilizing agent as well as any appended targeting/internalizing agents. For instance, Deng *et al*.[62] have shown (through NC size increase) that poloxamer 407 desorbs from paclitaxel NCs upon dilution. Another observation was that increasing the stabilizer-to-drug ratio resulted in poorer NC stability. Supported by evidence that higher concentrations of stabilizer led to the formation of micelles in addition to stabilizing NC coatings, the authors hypothesized that stabilizers deposited as unimers (*i.e.,* below the CMC) may have higher affinity to NCs than stabilizers deposited as multimers (*i.e.*, above the CMC), whose deposition process was in competition with micellization. This result could potentially attest to a different organization of the stabilizer on the surface of the NC. Indeed, owing to the complexity of systematically altering the structure of macromolecular stabilizing agents, few studies have attempted to rationally modulate interactions between the stabilizer and the NC for preventing desorption. Our group has recently presented a modular and systematic strategy for optimizing the affinity of polymeric stabilizers for NCs based on the post-polymerization modification of polymer precursors (containing α-propargyl-δ-valerolactone)[63] by thiol–yne click chemistry.[31] In this approach, two parent block copolymers of methoxy poly(ethylene glycol)-*b*-(α-propargyl-δ-valerolactone-*co*-ε-caprolactone) were used to create a library of 10 different stabilizers in which the hydrophobic polyester block was modified with alkanes of different length and structure. All

stabilizers had equivalent numbers of monomeric units and polydispersity indices to the parent stabilizers. Under the production conditions used, all stabilizers produced dense polymer brushes on the surface of the NC, and size-stability assays were found to strongly depend on the structure of the hydrophobic block.

In addition to altering the chemical structure of the stabilizer to promote interactions with the NCs, another approach is to cross-link the stabilizer around the NC and thus reduce shedding *via* physical entrapment. For instance, Kim and Lee[64] have electrostatically cross-linked chitosan on the surface of paclitaxel with tripolyphosphate, but have not evaluated the size stability of the NCs, nor the decrease of shedding achieved after cross-linking. Other electrostatically cross-linked stabilizers produced *via* the LbL deposition of polyelectrolytes are discussed in the following section. Our group has more recently designed block copolymer stabilizers that could be cross-linked directly on the surface of paclitaxel NCs by copper-catalyzed 1,3-dipolar cycloaddition to form nanocage–NC constructs.[65] Size-stability analysis showed that nanocages acted as sterically stabilizing barriers to NC–NC interactions and aggregation, which in turn imparted better size-stability to the NCs in comparison to the non-cross-linked coating. By dosing the amount of polymer released from nanocage–NC constructs, it was shown that the nanocages were 3–4-fold less shed from the NCs than comparable non-cross-linked stabilizers. In addition, transmission electron microscopy of the nanocages after complete dissolution of the drug NC revealed the intactness of the nanocage, demonstrating a successful cross-linking reaction (Figure 5A).

It should be noted, however, that shedding of the stabilizer may in fact be beneficial under certain circumstances. For instance, Liu *et al*.[57] have sought to exploit the shedding phenomenon by stabilizing 40 × 150 nm paclitaxel NC rods with TPGS. The rationale of this study was that

the tocopheryl-functionalized stabilizer may inhibit P-gp upon shedding, which may permit an enhanced treatment of multi-drug resistant cells. Indeed, the authors observed that in NCI/ADR-RES cells, which overexpress P-gp and are resistant to paclitaxel, NCs stabilized with TPGS exhibited a significantly enhanced anti-proliferative effect than free paclitaxel or paclitaxel NCs stabilized with poloxamer 407 (Figure 5B). The authors also observed that as the amount of TPGS increased compared to drug, the anti-proliferative effect increased for both TPGS-stabilized NCs and the physical mixture, indicating that TPGS modulated drug resistance transporters. Interestingly, however, at low TPGS concentrations, TPGS-stabilized NCs were more cytotoxic than the mixture, whereas at high surfactant concentrations they were comparable. These observations indicate that additional mechanistic investigations are warranted. In another example, our group has created amphiphilic block copolymer stabilizers that are spontaneously shed in response to a stimulus.[31] More specifically, the hydrophobic block of the stabilizer, responsible for physisorption on the investigated paclitaxel NCs, contained thioether groups that became substantially more hydrophilic in the presence of reactive oxygen species, thus driving the stabilizer from the NC and provoking its destabilization. Stabilizer shedding in areas of oxidative stress in the body, which are associated with a variety of diseases,[66, 67] may provide a means of provoking selective aggregation or promote uptake at these target locations, but should be tested *in vivo*.

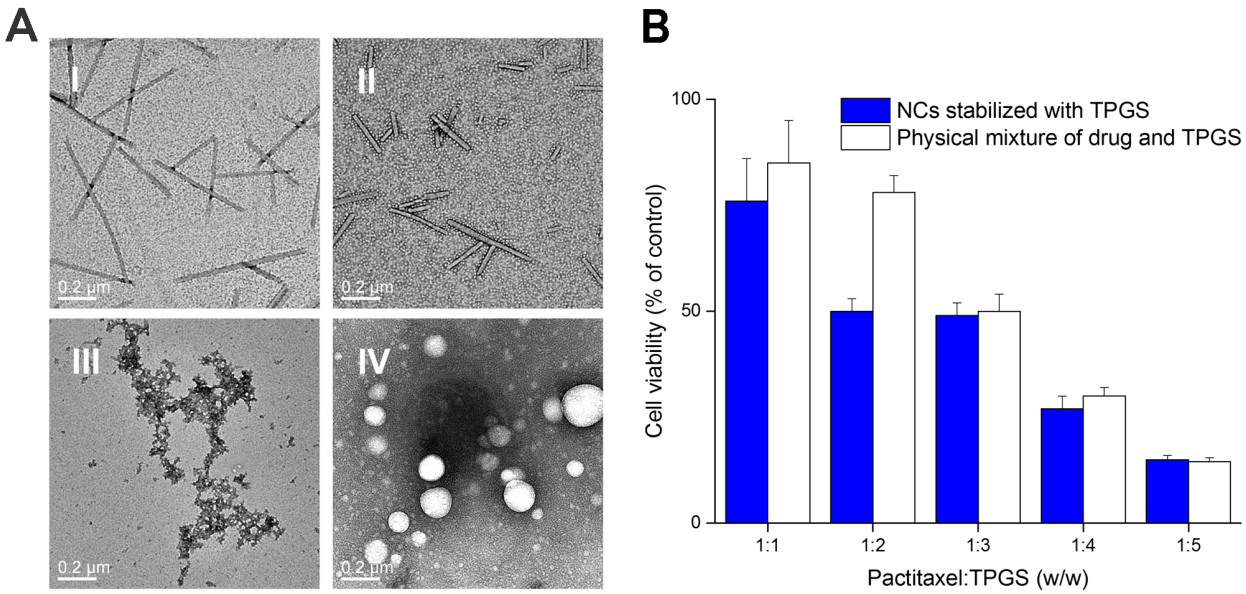

Figure 5. Controlling or exploiting stabilizer shedding. (A) Preventing NC stabilizers from shedding by cross-linking. Transmission electron microscopy images of paclitaxel NCs before (I) and after cross-linking (II). Following dissolution of the NCs from (I) and (II), an aggregate structure was observed for the non-cross-linked NCs (III) while intact polymeric coatings were observed for the correspondingly cross-linked NCs, in the form of discrete spheroids (IV). Reproduced from Fuhrmann *et al*.[65], with permission from American Chemical Society. (B) Stabilizer shedding promotes activity of NCs *in vitro*. Effects of paclitaxel/TPGS NCs (10 μM) with different amount of TPGS, in comparison to a physical mixture of paclitaxel and TPGS demonstrating that the "shed" stabilizer potentiates the activity of paclitaxel. Redrawn from Liu *et al*.[57], with permission from American Chemical Society.

Altering dissolution profiles (by means other than size)

It is clear from the examples above that size plays a key role in the dissolution characteristics of drug NCs, which alters their performance. Based on *in vivo* evidence, NCs with sizes above ca. 300–400 nm (depending on the specific drug in question) persist for a sufficiently long time that they could, in principle, accumulate passively within tumors *via* the EPR effect. However,

particles of this size may be subject to greater uptake by the mononuclear phagocyte system and would poorly diffuse in the extracellular tumoral matrix. Smaller NCs may have greater abilities to penetrate tumors, but their targeting is more challenging because dissolution must be delayed. Several approaches have been examined for this purpose.

Drug NCs can be re-processed following miniaturization to alter dissolution kinetics. For instance, paclitaxel NCs could be re-nanozised by an incubation–sonication technique.[62] In this technique, the authors incubated NCs at 37 ºC for a certain period during which time NC size increased via ripening processes. This was then followed by sonication to break the growing NCs into smaller ones. The authors observed that the re-nanosized NCs displayed significantly greater size stability, which they attributed to the disruption of the preferred growth pattern of the NCs. These interesting findings should be pursued with analysis of dissolution kinetics under sink conditions and a more in-depth characterization of this phenomenon. Lu *et al*. also produced very stable 300-nm NCs of paclitaxel by adsorption of transferrin. The NCs did not exhibit a size change during the 3 months study period. The increased stability compared to the bare NCs was also reflected in a slightly slower drug release during dissolution experiments.[68]

One of the most investigated systematic approaches for altering microparticle[69, 70] and, more recently, NC dissolution kinetics *via* stabilizing coatings produced by LbL assembly of polyeletrolytes. In this technique, the hydrophobic drug NC is first covered by an anchoring layer typically composed of a small molecule amphiphile and a polymer, and is followed by the sequential deposition of multiple layers of charged polyelectrolytes. Model experiments have shown these coatings are semi-permeable (*i.e.,* permeable to small molecules smaller than specific cut-offs),[71] which points to the importance of well characterizing the coating. Despite this semi-permeability, an effect on dissolution rate of NCs, albeit a small one, has been

observed. For instance, the rate of drug release from 300-nm paclitaxel NCs was independent of the thickness of the stabilizing coating, when this coating was thinner than 3.5 bilayers of poly-L-lysine and sodium heparin (Figure 6).[72] However, when the number of bilayers increased from 4 to 12, a slight decrease in the drug release rate was observed. Agarwal *et al*.[30] have observed a marginal difference in the rate of dissolution of 125-nm tamoxifen NCs stabilized with either 0.5 or 3 bilayers of poly(dimethyldiallylamide ammonium chloride) /poly(styrene sulfonate). One particularly interesting feature of the LbL approach is the large parameter space available for constructing these stabilizing coatings including: polyelectrotyte type/architecture/molecular weight, addition of salts and other additives, anchoring layer chemistry. Future work should focus on assessing how these parameters inflence the semi-permeability of LbL-assembled coatings, which may provide the means to rationally alter NC dissolution.

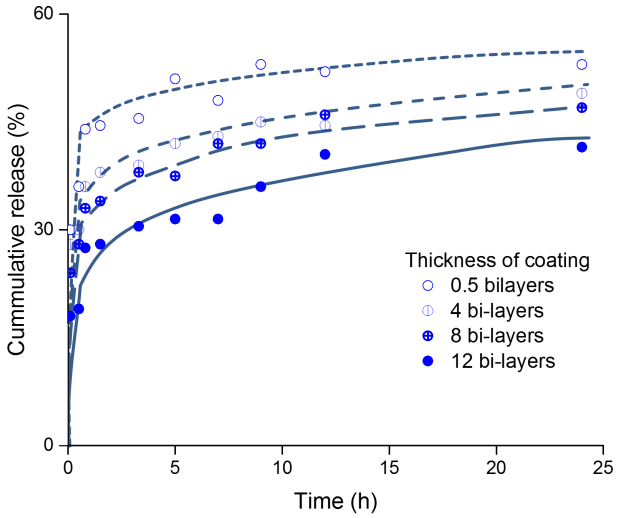

Figure 6. Altered NC dissolution *via* the stabilizing coating. Paclitaxel release from 300 nm NCs coated with (poly-L-lysine/heparin)$_n$ shells. Redrawn from Shutava *et al*.[72], with permission from Royal Society of Chemistry.

Outlook

The studies presented above suggest that drug NCs may in the future play an important role in targeting significant amounts of drug to sites of disease. NC size can for all intents and purposes be selected using (universal) preparation approaches – NC persistence *in vitro*/*in vivo* strongly correlates with size – and cellular uptake and tumor accumulation can be promoted by modifying the surface chemistry of the coating. While these aspects have been individually demonstrated in the examples discussed herein, future research should question how to overcome the challenges associated with assembling these individual properties into efficient and optimized therapeutics. For instance, as small (sub-100 nm) nano-carriers are known to penetrate even poorly permeable tumors,[73, 74] could such an effect also be achieved in practice with NCs? Can the rate of drug release of these smaller, dissolution prone NCs be controlled by their stabilizing coating? Can stimuli-responsive stabilizers provide a means for selective NC destabilization and accumulation at sites of disease? In addition, other forms of targeting strategies *in vivo* could be foreseen. For instance, it has been recently reported that composite nanoparticles of a gemcitabine prodrug and magnetite yielded enhanced tumor accumulation and therapeutic activity *via* magnet-assisted targeting.[75] Finally, other opportunities for enhancing the targeting potential of NCs should be explored and which are not part of the NC construct itself. Sugahara *et al*.[76] have indeed recently demonstrated that tumor-penetrating peptides, co-administered with Abraxane®, increased vascular and tissue permeability leading to a 12-fold increase of the tumor accumulation *versus* in the absence of peptide. This area is ripe for discovery and answering these questions will require creative new hypotheses to be tested.

Acknowledgments

Our group's work on NCs has been supported by the Swiss National Science Foundation (310030_138342).

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