EXPERT OPINION

Hybrid nanocrystals: University of Kentucky US20060280680A1

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This patent application claims an interesting and novel combination of passive accumulation of drug nanocrystals within diseased tissue, in combination with active uptake of the nanocrystals by diseased cells. The patent application further claims the hybrid nanocrystals combining imaging or stabilizing molecules as inclusions in the crystal matrix. There is a focus on cancer chemotherapy and imaging, but the initial claims are not disease specific. In this patent evaluation, the novelty and utility of this application is examined, while the state of the art in nanocrystal formulations and formulation is discussed.

Keywords: cancer, imaging, nanocrystal, targeted drug delivery, theranostic

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1. Introduction

In recent years, an increasing number of lead compounds identified as highly active toward specific molecular targets are abandoned because of their unfavorable dissolution profile or solubility properties [1,2]. Poor active pharmaceutical ingredient (API) dissolution and solubility suggests poor delivery prospects particularly for the oral route, and attempts are typically made to achieve more favorable physicochemical properties through chemical derivation [3]. Some success has been achieved in finding delivery strategies to circumvent the abandonment of these molecules and bring more of these compounds back from the bench to the bedside of patients, particularly through the use of nanotechnology [4-6]. Several nanoparticle systems have reached the clinic or preclinical testing, including Doxil®/Caelyx®, Myocet®, Ambisome®, Amphotech® [7], PEG-Intron®, PROTHECAN®, Neulasta®, Macugen®, NK911, Xyotax®, Mylotarg®, Tositumomad® and others [8].

Nanoparticles for drug delivery come in several varieties, including polymers (including dedrimers), liposomes, micellar structures, proteins, carbon nanotubes and nanocrystals [5,8-11]. Each of these nanomaterials represents an entire field of study in nanoparticle science. We will focus on nanocrystalline – and incorporate into that description amorphous drug nanoparticles – materials, as it is the most relevant field of study for this patent application [12]. Of the nanocrystals described, several of these technologies have reached the approval for clinical dosage and many others have been in clinical trials. Rapamune® and Emend®, rapamycin and aprepitant crystals for oral delivery [13,14], and Abraxane®, albumin-coated paclitaxel [15,16] were some of the first to reach the market. An increasing number of systems now exist for delivery for various diseases focusing on oral uptake enhancement, due to the knowledge that smaller particle size increases dissolution rate [17] and observed solubility [18]. Many of these systems appear on the verge of clinical trials [19-21].

Nanocrystal technologies have the potential to be the most efficient way to package APIs into nanoparticles because almost the entire particle can comprise the API. There are two broad approaches to forming nanocrystals. The first, top-down approach, which starts from a large crystal and reduces its size to nanoscale through

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milling techniques; patented examples of this approach are Hydrosol [22-24], NanoCrystal® [13], DissoCubes® [25] and NANOEDGE® [26]. The second, bottom-up approach, grows crystals from a solution limiting the growth to the nanoscale. This is classically achieved by controlled precipitation where the API is dissolved in a solvent then atomized and sprayed into an antisolvent that instigates precipitation in the droplets [22,24] but other bottom-up approaches exist [27-30]. Conventional bottom-up approaches creating nanocrystals have proven difficult to scale-up in pharmaceutical industry due to the strict control necessary to successfully carry the process out [19]. Regardless of the methods of production, nanocrystals have been focused primarily toward oral administration, but intravenous and other parenteral routes have been investigated clinically and approved with cancer being a primary disease for parenteral administration.

The focus of the patent application being examined is intravenous administration and local targeting [12]. Targeted delivery is not typically possible for orally administered agents. Tumor targeting has been achieved by both passive accumulation and active targeting mechanisms [31]. Passive accumulation of nanoparticles within tumor stroma makes use of an effect described as the enhanced permeability and retention effect (EPR) [32-34]. Four differences between tumor and non-tumor tissues are responsible for this phenomenon: i) the hypervascularity of tumor tissue; ii) hyperpermeability of tumor vasculature; iii) defective architecture of tumor vascular and iv) less efficacious lymphatic drainage due to abnormalities caused by the tumor. Although EPR is predominantly described for tumors, many inflamed or diseased tissues exhibit one or more of these characteristics [35-38] leading to some preference for the term passive accumulation and not passive targeting [39]. Active targeting, on the other hand, makes use of tumor-specific membrane-bound receptors that have affinity to a ligand or antibody, respectively, on the surface of the nanoparticles [31]. Active targeting typically does not alter the distribution of nanoparticles to a diseased tissue, but once the nanoparticle has distributed to the tissue, the active binding improves retention and can augment or cause cell entry for nanoparticles that would not otherwise enter cells. Nanoparticles can, therefore, be engineered to deliver specific therapy by incorporating tumor-specific ligands or antibodies on the surface of the particle without interfering with the chemical structure of the bulk of the drug itself. Though the targeted nanosystems are mostly in preclinical stages, several targeted chemotherapeutics utilizing targets such as the folate receptor [40,41], CD33 [42] and VEGFR [43] are currently in clinical trials.

The patent application of interest claims nanocrystals formed from poorly soluble, chemotherapeutic drugs, specifically doxorubicin, camptothecan and paclitaxel, incorporating tumor-targeting ligands or antibodies into the crystal that instigate receptor-mediated endocytosis to treat malignant, solid tumors [12]. The patent application further extends its claims to radionuclides or imaging agents to combine cancer therapy and diagnostic imaging in an emerging field known as theranostics. It is of note that the key difference between this patent application and previous patent applications is that this one is for actively targeted and hybrid nanocrystals, and that, herein, lies the novelty of this invention.

2. Chemistry

Novel chemistry or chemical entities are not claimed as part of this patent application. Even the physical chemical methods used to create the nanocrystal system claimed in this patent application are not claimed to be novel. It is likely there are other methods to create nanocrystals that would arrive at the claimed system as efficiently as the examples described in the application and the previously described technologies for nanocrystal production. The patent application focuses on the solid state interactions of small molecule drugs, within a nanocrystal, and one of a group of hybridizing agents that imparts i) targeting and/or uptake and ii) imaging capability.

It is widely accepted that a greater and greater proportion of new chemical entities (NCEs) and APIs are poorly soluble in water and poorly permeable to intestinal uptake: the so-called class IV drugs [44]. The patent being examined does not discuss specific chemistries used for producing poorly soluble drugs, but will allow any new low solubility, low permeability molecule to be delivered via the intravenous and other parenteral routes. Since specific chemistries are not specified, the physical chemistry and technologies involved in particle production will be the focus of the chemistry assessment.

As previously stated, there are two general approaches: a top-down approach and a bottom-up approach (**Figure 1**), with the top-down approach generally yielding higher numbers of usable crystals. More specifically, there are three primary methods of top-down nanocrystal creation: bead/pearl milling, high pressure homogenization and certain combination technologies. Bead/pearl milling involves a bead/pearl mill to get particle size reduction from larger crystals. The force of shearing generated from the motion of the milling media leads to the particle size reduction. Particle reduction time depends on several factors, including the characteristics of the API, the size of the milling media, the amount of surfactant used and the temperature. The main problem with this method is that the milling material can become eroded through the process. However, typical industrial scale production is possible with this Food and Drug Administration (FDA) approved method.

High pressure homogenization is based on the jet-stream principle or piston-gap homogenization based in water or other non-aqueous media. Both of these methods lead to particle collision and shear forces to aid in micronization. The microfluidizer technology is an example of the jet-stream principle. This method is used to obtain submicron particle size of poorly soluble drugs and often requires several cycles to obtain sufficient particle size reduction. Both the DissoCubes [25] and the Nanopure® technologies are based

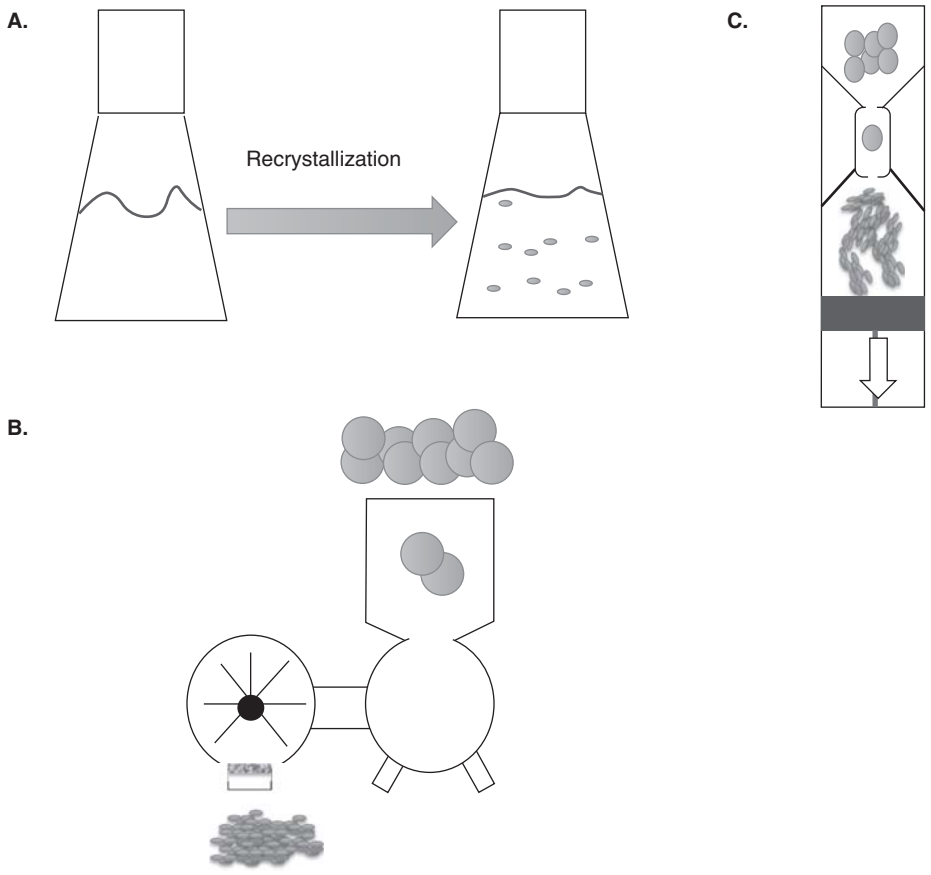

Figure 1. Schematic of top-down and bottom-up nanocrystal production. (A) Schematic of crystallization or bottom-up method of production. Top-down methods include **(B)** milling and **(C)** high pressure homogenization.

on piston-gap homogenization. The DissoCubes technology is based in aqueous media. The drug is dispersed in this media and put under pressure by a piston. The cavitation that results produces shockwaves which aid in drug submicronization. Particles formed by this method are crystalline and cuboid or irregular in shape. Finally, the Nanopure technology uses a non-aqueous primary dispersion medium. Particle size reduction is due to shear forces and particle collision. Low temperatures can be used with this method, which is advantageous with temperature-sensitive drugs.

Combination technologies of the top-down approaches combine classical precipitation steps followed by a high energy step [26]. One problem with combination technologies is that often times, organic solvent is used to precipitate the nanocrystals out of solution, and removal of this solvent needs to be done aseptically, which can become expensive and tedious on the industrial scale. Each of the methodologies for formation of nanocrystals has advantages and disadvantages that have been extensively reviewed and detailed descriptions are beyond the scope of this discussion [5,19-21,27,45-56].

The nanocrystal claimed in this patent utilized as proof of concept for the state of matter was created with a bottom-up approach. The drug was dissolved in a favorable solvent and subsequently precipitated using another solvent to get crystalline nanoparticles. Crystal growth can be difficult to control with strict temperature and environmental controls are needed. The number of variables to control has made this a difficult method to use on commercial products, and there are currently no available pharmaceutical products available. Originally developed by List and Sucker [22-24], the drug must be soluble in at least one of the solvents and there is the issue of solvent retention in the crystal as inclusions, but these types of inclusions are precisely the key to this invention.

Inclusions are well known in crystals [57], with inclusions accounting for much of the variability in color and clarity in commodity crystals. Inclusions can range from a single atom to supramolecular in nature. Inclusions can be conceptualized as the insects that can be found encased in amber for many centuries; however, it should be noted that amber is amorphous in nature not crystalline. From this conceptualization, it is clear that molecules of varying size can be placed within a structure of larger size without being a true part of the crystal, **Figure 2**. For crystalline materials, the order of the crystal is lost in the region of the inclusion and the ability for the molecule to be retained is a function of the energetics of interaction between the crystal and the included substance

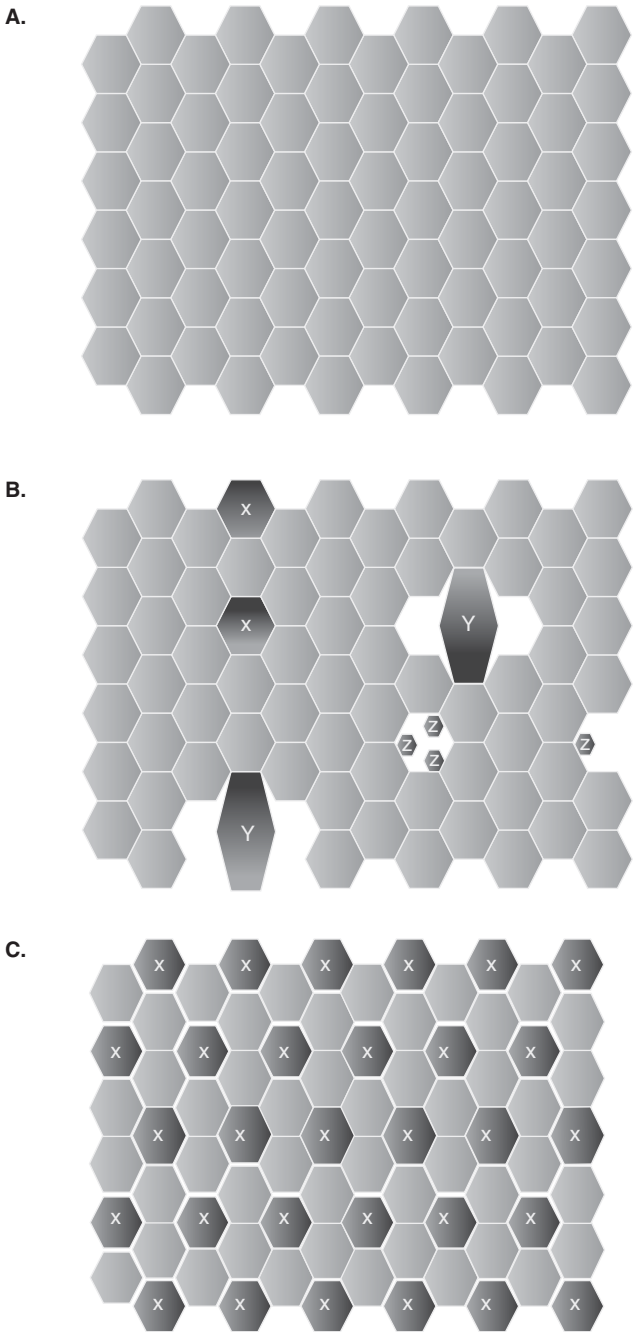

Figure 2. Two-dimensional schematic description of the possible inclusions within or on the surface of a crystal. (A) Crystal lattice with no inclusions. **(B)** Random inclusions in a crystal lattice. Inclusion may be similar in size and shape (X), significantly larger than the host molecule (Y), or much smaller than the host molecule (Z). Note that there is expected to be some binding similar to that which forms the structure of the pure crystal lattice. **(C)** Co-crystal lattice.

and the rate of growth (or dissolution) of the crystal. The crystal would be expected to enclose an inclusion after sufficient growth has occurred to cover the defect. One type of inclusion, the co-crystal, has stoichiometric inclusion of at least one additional molecule in the structure of the crystal [58-60]. If this were the case, **Figure 2C**, the co-crystallized component would be regularly included and not randomly, **Figure 2B**. For most in the pharmaceutical industry, the non-stoichiometric inclusion within a crystal is a flaw. Unlike most, the inventors of this patent application have taken the, probably non-stoichiometric, inclusion as a positive for therapy and not as a flaw. This is an opportunity to include a small amount of active substance within the crystal allowing multiple avenues of therapy.

3. Biology and action

The primary claim, on which all other claims are dependent within the patent application, is described (Figure 2 of patent application [12]) and supported by the single figure (Figure 6 of the patent application [12]), wherein a ligand, folate in the examples, specific to a cell surface receptor, folate receptor in the same examples, is incorporated as inclusions in the nanocrystals. In this figure (Figure 6 of patent application [12]), it is clear that an elevated level of uptake is present when the ligand is incorporated as an inclusion. In this case, the ligand and a fluorescent dye appear to be included in the nanocrystals, that is, crystals internalize in the cells. The proposed mechanisms of uptake are either i) the dissolution of the ligand prompting cellular uptake via any of several endocytotic mechanisms or ii) direct binding of the ligand to the receptors in the cell membrane [61-64]. The data presented do not differentiate either of the two mechanisms of uptake nor does the patent claim either of the two mechanisms of uptake specifically or preferentially. The patent claims either and both of the two biologic uptake mechanisms. To our knowledge, this is a novel and creative idea that has not been published to date, including by the inventor of this technology. Since, although the utility is shown, there is not substantial data shown for the specific mechanism of uptake, we will focus further discussion toward data on the biologic function which has been published within and beyond the patent and supporting information beyond this inventor.

From this inventor, two recent publications related to the nanocrystal technology have been identified, but neither has clearly utilized the claimed combination of drug and targeting agent which is the primary claim of the application and all other dependent claims [12]. Both publications have used cancer chemotherapeutic nanocrystals. The first utilized camptothecan nanocrystals [29] and the second paclitaxel crystals [65]. The paclitaxel nanocrystals were combined with imaging agents.

At the same dose, pure camptothecan nanocrystals were shown more effective than camptothecan solution (DMSO) toward the MCF-7 breast tumor cell line [29]. Cellular uptake of camptothecan nanocrystals was found to be increased in several other lines, including the MCF-7 line. Cellular uptake

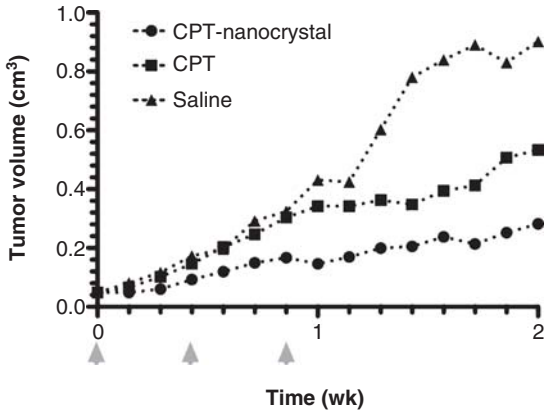

Figure 3. Camptothecin (CPT) nanocrystals inhibit tumor growth. Saline treated (▲), CPT solution (■), and CPT nanocrystals (●) all exhibit increased tumor size over 2 weeks after administration of the treatments on days 0, 3 and 6 (gray arrowheads).
The data were extracted from Figure 5 of [29].

was ablated by chlorpromazine, an inhibitor of clathrin-mediated endocytosis, suggesting an active uptake of the drug crystals. Taken together, these data demonstrate the ability of cells to take up nanocrystals with at least one cell nanocrystal formulation superior to a conventional solution. This utility was further corroborated *in vivo* using BALB/c mice that had MCF-7 tumors implanted subcutaneously. These animals were treated with 7.5 mg/kg camptothecan either as nanocrystals, propylene glycol solution or a saline control. Significant tumor growth inhibition was noted for the camptothecan solution and the camptothecan nanocrystals, with the latter suppressing growth most efficiently (**Figure 3**). It is notable that these nanocrystals were not actively targeted to the tumor, but relied on EPR for passive accumulation and success was from clear biodistribution in which significantly more camptothecan accumulated in the tumor compared with camptothecan solution 1 day after administration.

Similarly, hybrid paclitaxel nanocrystals with fluorophore, FR-749 or rhodamine, integrated as random inclusions were created by solvent/antisolvent precipitation and used to treat mice subcutaneously implanted with bilateral MCF-7 tumors [65]. Imaging was then used to examine nanocrystal localization in the tumor by the enhanced permeation and retention effect. Tumor localization by 24 – 48 h post i.v. treatment of 20 mg/kg paclitaxel nanocrystals was observed where local imaging enhancement was not present in controls. Overall, the results of this study highlight the utility of hybrid crystals in diagnostic therapy, or theranostics, and make viable the claim that hybrid nanocrystals can be used for this purpose.

The published *in vivo* results show successful nanocrystal creation from two of the three poorly soluble chemotherapeutics specifically claimed by the patent application. Furthermore, one of the studies shows successful creation of hybrid nanocrystals by combining chemotherapeutic with a fluorophore for imaging studies. Importantly, both published studies rely on passive targeting by EPR to localize nanocrystals to the tumor.

4. Expert opinion

Nanotechnology has provided the drug delivery world with an arsenal which it can use to deliver compounds deemed unlikely or unfavorable drug candidates. Several nanoparticle systems are already on the market [20], and each year more trials commence furthering the development of these technologies [5,66]. Engineered nanoparticles provide several potential advantages over traditional drug delivery including exceptional loading, that is, 100% of particle is drug, and customization of particle size. The size of the particles influences how a particle distributes influencing the clearance and elimination rates. For cancers, there is clear correlation between size and penetration into solid tumors [34]. Historically, the idea of intravenous particle injection was not readily accepted, but the benefits of particulate systems are being rapidly accepted.

The crystallization method used by the authors and outlined in the introduction of this patent to create nanocrystals has proven to be difficult to scale to industrial production because of complexity for controlling particle size [21]. However, the inventor does not claim the method in this patent application [12]. Indeed, the authors claim no methodology in the patent, only the final state of matter, nanocrystal with integrated ligand and further claims including imaging. Though this in no way bears on the claims of the patent, it is likely that before trials are able to begin, a more scalable method for producing hybrid nanocrystals will be applied, and only slight modifications of current methods will likely succeed in being able to generate crystals with engineered impurities.

This patent for hybrid nanocrystals moves nanotechnology a step further by actively targeting both an API and diagnostic markers to sites of interest. Data supporting the patent demonstrate the ability of hybrid nanocrystals to be useful both in diagnostic and therapeutic effect. While these particles have not been actively targeted to the tumor, passive accumulation provides proof-of-principle of the utility of the particles. Active cellular uptake was shown in cell culture, but further proof of cell uptake is necessary *in vivo*. It is important to note that despite the reported data supporting the ideas of the inventors, no identified publications explicitly support the ideas protected under the claims of the patent application [12]. That is, there are limited data supporting that incorporated targeting agents increase cellular uptake *in vivo* or *in vitro*, beyond the single figure of the patent application [12]. As a patent application, further modifications of the claims are expected and it is possible that these claims are added or additional provisional patents have been submitted. This does not diminish the novelty or importance of the claims of the application [12]. Further, it is clear that the utility and scope

of the patent includes not only active targeting but also theranostics applications. The first steps in the process of validating the technology were to show that the particles were therapeutic and this has been clearly achieved [29,65].

The next step in validating the technology focuses on the ability of ligands to further increase efficacy and to confirm cellular uptake *in vitro* and *in vivo*. The amount of this additional intratumoral accumulation achieved in an actively targeted system will depend on how these ligands mediate interactions between the nanocrystal and target cells. Either the ligand will remain intact as part of the crystal, and the entire crystal will interact with the cellular target, or the ligand will dissolve from the crystal and interact with its cellular target independent of the crystal. Either of these mechanisms may induce endocytosis of the crystal, but distinguishing which mechanism predominates will be necessary to choose effective ligands for a particular system.

For small molecular ligands, it is our opinion that there is limited potential for direct interactions with cellular receptors while part of the crystal. This is due to the interactions that typically stabilize the molecule in the crystal are the same types of interactions that allow ligand binding. This will depend greatly on the orientation and specific ligand. For larger ligands such as proteins, there is much greater possibility of direct signaling, but there is more difficulty in integrating the protein into the small molecule structure, particularly for a particle (~ 200 nm) not much larger than an average protein (~ 10 nm). It is our opinion that the more likely mechanism of active uptake would be the stimulation of endocytosis by released molecules. We will be watching the literature for further evidence and confirmation of one mechanism or the other.

Declaration of interest

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