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Review article

State of the art of nanocrystals – Special features, production, nanotoxicology aspects and intracellular delivery

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ABSTRACT

Drug nanocrystals are the latest, broadly introduced nanoparticulate carrier to the pharmaceutical market from the year 2000 onwards. The special features of nanocrystals for the delivery of poorly soluble drugs are briefly reviewed (saturation solubility, dissolution velocity, adhesiveness). The industrially relevant bottom up (precipitation) and top down production technologies (pearl milling, high pressure homogenization, combination technologies) are presented. As nanotoxicological aspects, the effect of size, degradability versus biopersistency and intracellular uptake are discussed, classifying the nanocrystals in the low/non-risk group. Intracellular uptake plays a minor or no role for dermal and oral nanocrystals, but it plays a key role for intravenously injected nanocrystals (e.g. nevirapine, paclitaxel, itraconazole). Uptake by the macrophages of the mononuclear phagocytic system (MPS, liver spleen) can modify/optimize blood profiles via prolonged release from the MPS (itraconazole), but also target toxicity by too high organ concentrations and thus cause nanotoxicity. The balance in the competitive intracellular uptake by MPS and the target cells (e.g. blood–brain barrier) decides about therapeutic efficiency. The concept of "differential protein adsorption" to modulate this balance is shown for its applicability to nanocrystals for intracellular delivery to the cells of the blood–brain barrier (atovaquone).

1. Introduction

Drug nanocrystals are particles made from 100% drug; typically, they are stabilized by surfactants or polymeric steric stabilizers [1,2]. Hence, these particles possess a 100% drug loading in contrast to matrix nanoparticles consisting e.g. of a polymeric matrix (polymeric nanoparticles [3] or a lipidic matrix (nanoemulsions [4,5], liposomes [6,7] and lipid nanoparticles [8]) (Fig. 1). The high loading makes them very efficient in transporting drug to or into cells, reaching a sufficiently high therapeutic concentration for the pharmacological effect. The nanocrystals are typically produced in a liquid dispersion medium, i.e. the nanocrystals are suspended in the liquid (=nanosuspensions).

The nanocrystals were invented at the beginning of the 1990s [9–11]. The first products appeared very fast on the market from the year 2000 onwards. Despite one exeption, all marketed products by now are for oral administration; they are all dry dosage forms (tablets, capsules), and only one product is a suspension (Megace ES). After oral administration, the nanocrystals are dissolving in the intestinal tract. There is no or negligible uptake of particles from the gut. In general, the uptake of intact nanoparticles from the gut via the cellular, paracellular or lymphatic route was found to be very low [12] and is therefore by now not exploitable for therapy. Nanocrystals are also exploited in dermal products [13,14], but also in this case they rather dissolve on the skin, and the drug molecules penetrate into the skin. Practically, no investigations are published by now about a potential uptake by cells after oral administration or dermal application.

In contrast, uptake by cells plays a key role in intravenous injection of aqueous nanosuspensions. These interactions decide, if a product can be formulated successfully, or if the development "dies". Aqueous nanosuspensions for i.v. injection are in development for two purposes:

1. to reduce the side effects of existing intravenous products and
2. to target drugs specifically to certain sites, e.g. brain targeting.

For both purposes, the uptake by the cells of the mononuclear phagocytic system (MPS) is a crucial factor, examples discussed are paclitaxel and itraconazole. For targeting to a specific site, the nanocrystals need to bind to the surface of the target cells and then

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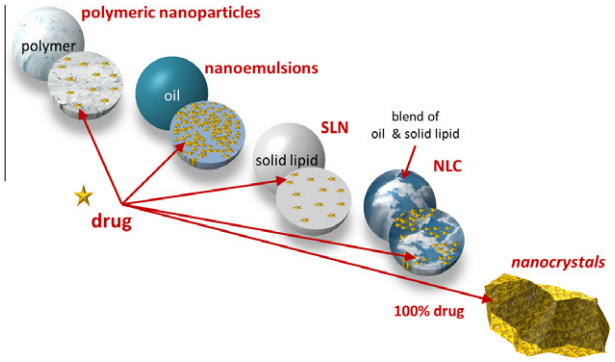

Fig. 1. Basic structure of polymeric nanoparticles, nanoemulsions, solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) (=all matrix particles) versus drug nanocrystals. The SLN are made from a solid lipid only, the NLC from a blend of a solid and a liquid lipid (oil), but both being solid at body temperature. The matrix particles have drug distributed throughout the matrix and/or adsorbed onto their surface (drug loading ≪100%); the nanocrystals consist of 100% drug. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

to be internalized to deliver the drug. Classical examples for the delivery of drugs to the endothelial cells of the blood–brain barrier (BBB) are discussed, e.g. dalargin, paclitaxel and atovaquone. For a better understanding of the nanocrystals, their production and special features are very briefly described, before discussing the interaction with the cells, including nanotoxicology aspects.

2. Special features

Nanocrystals are made from poorly soluble drugs; water soluble drugs cannot be formulated as a nanocrystal (at least not in aqueous dispersion medium). Formulating poorly soluble drugs as nanocrystals can solve their biopharmaceutical delivery problems, e.g.

- too low bioavailability after oral administration,
- too low penetration into the skin (low dermal bioavailability),
- too large injection volume for i.v. administration, and
- undesired side effects after intravenous injection when using traditional formulations (e.g. solutions with solubilized drug).

This is possible due to the special features of drug nanocrystals:

1. increased saturation velocity,
2. increased dissolution velocity, and
3. increased adhesiveness to surfaces/cell membranes

compared to the micro-sized drug powders. These features occur, because the transfer of particles from the macrosize range to the nanodimension changes their physico-chemical properties. This is the basis of nanotechnology. For a detailed description of the physical background of these effects (e.g. equations by Kelvin, Noyes–Whitney, Prandtl, Ostwald–Freundlich), it is referred to [15–17]. The special features of the nanocrystals are summarized in Fig. 2.

The oral bioavailability of poorly soluble drugs can be increased when it is limited by their dissolution velocity and too low solubility (class II drugs of the biopharmaceutical classification system (BCS) after Amidon [18]). Also the penetration into the skin increases due to the increase in the saturation solubility, leading to a larger concentration gradient between dermal cream with nanocrystals and skin. The larger gradient promotes penetration. In case drugs are poorly soluble, injection volumes are consequently typically too large (e.g. a few 100 ml or even several liters). As alternative, the drug can be injected as aqueous isotonic nanosuspension, e.g. up to 10% drug content (=injection of 1 g drug in 10 ml volume). Higher concentrates are also possible if required [19]. Some poorly soluble drugs can be injected in a sufficiently small volume when solubilizing them with surfactants (e.g. paclitaxel with Cremophor EL) or complexing them with cyclodextrins (e.g. itraconazole with hydroxypropylcyclodextrin, HP-CD). The Cremophor can cause anaphylactic shock when administering paclitaxel [20]. The HP-CD is held responsible for nephrotoxicity as side effect of the commercial i.v. product Sporanox [21,22]. This can be avoided, when replacing these formulations by nanosuspensions stabilized with intravenously well tolerated surfactants/stabilizers such as Tween 80 or Poloxamer 188. This is discussed below.

3. Production

3.1. Bottom up technologies

There are two basic approaches to produce drug nanocrystals, the bottom up and the top down technologies. In the bottom up processes, one starts from the molecule in solution, the molecules are aggregated to from particles, being crystalline or amorphous. It is a classical precipitation process (in latin: via humida paratum, prepared in a wet process). The basic principle is that the drug is dissolved in a solvent, the solvent solution is then added to a non-solvent, as a consequence the drug precipitates. Crucial in this process is it to control the structure of the particles (amorphous versus crystalline) and to avoid growth of the crystals to the μm size range.

The industrially relevant technologies are various precipitation processes developed by Auweter, Horn and co-workers, the patents belonging to BASF. They describe e.g. precipitated water-insoluble colorants in colloid-disperse form [23], the production of carotenoid preparations in the form of cold-water dispersible powders [24,25] and are exploited in products for food and soft drink industry, e.g. in the product Lucarotin® 10 CWD from BASF, where CWD stands for cold-water dispersible. The precipitation process can be run this way that amorphous nanoparticles result. This technology is exploited by the company Soliqs/Ludwigshafen (previously Knoll, belonging to BASF) to produce Nanomorph™, amorphous drug nanoparticles. They have a better solubility than crystalline nanoparticles because in general the solubility of amorphous material is higher. Another process leading to crystalline nanoparticles was developed by Sucker et al., the so-called hydrosols [26]. This IP belongs to Novartis, but has not yet been exploited in products to our knowledge.

There are various other bottom up technologies, e.g. the high-gravity controlled precipitation technology, sonocrystallization, confined impinging liquid jet precipitation and multi-inlet vortex mixing, for a detailed review it is referred to [27]. A basic disadvantage of many precipitation processes is the use of organic solvents, which need to be removed again in most cases, increasing the costs. Especially when large solvent volumes are required, being the case when the drug exhibits low solubility in water and also in organic solvents. Therefore, for pharmaceutical industry, typically the top down technologies are employed for the products introduced to the market.

3.2. Top down technologies

In the top down technologies, one starts from large crystals in the μm range and goes down to the nanodimension by diminuting the crystals, i.e. performing a milling process. Dry milling (e.g. jet milling) is not efficient to obtain a size in the nm range; therefore, wet milling is applied. Wet milling means that the drug particles

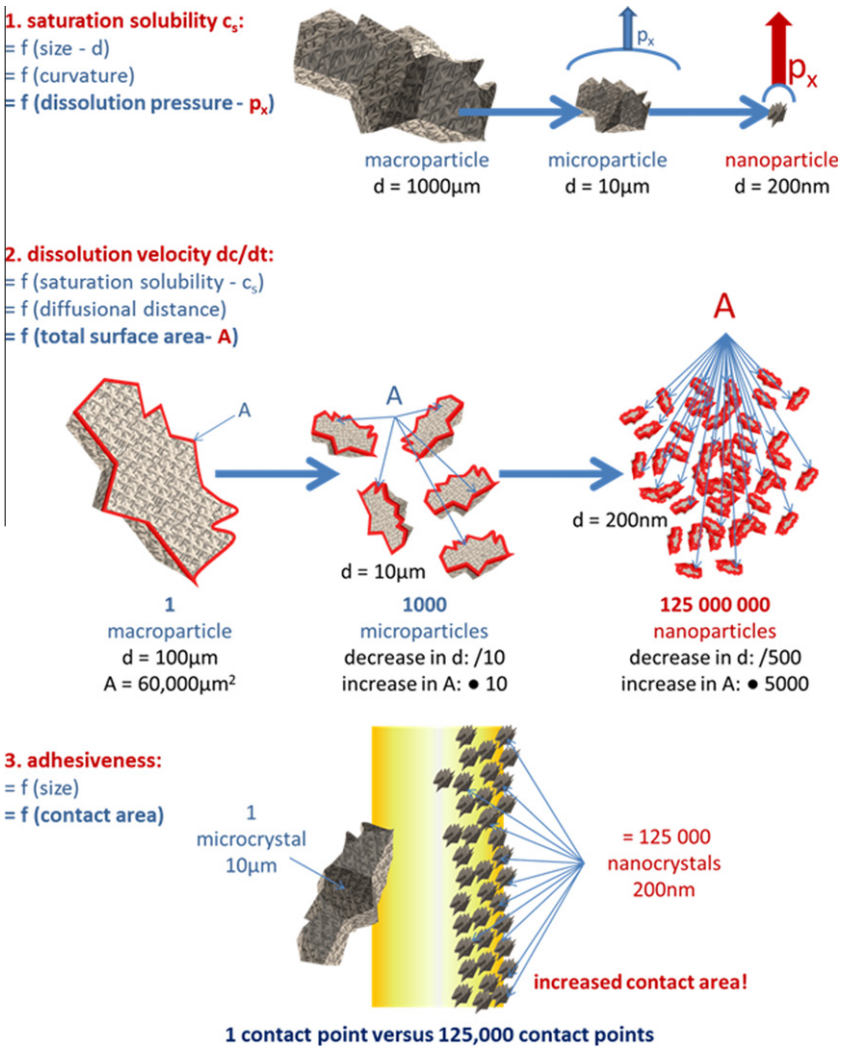

Fig. 2. Features of nanocrystals: increased saturation solubility due to increased dissolution pressure of strongly curved small nanocrystals (upper), increased dissolution velocity due to increased surface area (middle), and increased adhesiveness of nanomaterial due to increased contact area of small versus large particles (at identical total particle mass), for surface: calculations were performed as cubes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

are dispersed in a surfactant/stabilizer solution; the obtained macrosuspension is then subjected to milling energy.

A low energy milling process is the pearl mill (bead mill), the technology developed by Liversidge and co-workers [28,29], being the NanoCrystal™ technology of élan (prev. Nanosystems). Almost all products on the market are produced with this technology. The suspension is added to a milling container containing milling pearls, typically in a size of 0.2 mm or 0.4–0.6 mm. The pearls are moved by an agitator, the crystals are ground between the moving pearls, and the resulting product is a nanosuspension.

Alternatively, the crystals can be ground by a high energy process using high pressure homogenization. The Canadian company RTP developed a process using a jet stream homogenizer, e.g. the Microfluidizer. The high energy fluid streams of the suspension collide; in the collision zone, the crystals are diminuted by collision and cavitation [11]. The technology is called IDD (insoluble drug delivery) technology [30]. This technology and the company RTP were later acquired by SkyePharma PLC (now SkyePharma Canada).

An alternative high energy method is the use of piston-gap homogenizers, developed by Müller and co-workers [31]. The suspension passes with a high velocity (e.g. 500 m/s) a small gap, e.g. 10 µm in height. In the gap, the crystals are diminuted by cavitation, collision of crystals with each other and the steel wall, and shear forces of the liquid. Trade name of this technology is DissoCubes®. Typical production conditions are 1500 bar and up to 20 passes through the high pressure homogenizer (e.g. homogenizers from APV, Gaulin and Avestin). Also this technology was acquired by SkyePharma (year 1999). All these homogenization processes are performed with water suspensions. A follow-up development was the homogenization in water-free media (e.g. oils, liquid polyethylene glycols (PEGs)) or water-reduced media (e.g. glycerol/water mixtures for the production of isotonic formulations) by PharmaSol/Berlin (Nanopure®) [31]. Oil dispersions can directly be filled into gelatine capsules for oral administration, or injected parenterally as controlled drug delivery depot.

3.3. Combination technologies

The combination technologies combine generally a pre-treatment step followed by a high energy process. Baxter developed the NANOEDGE™ technology. In the first step, crystals are precipitated, and the obtained suspension is then subjected to a high energy process, typically high pressure homogenization [32]. The top

down technologies have the advantage, i.e.no solvents are needed. The Baxter technology looses this advantage by having a precipitation pre-step. Advantage of this combination is that it provides a certain freedom to operate in view of other existing intellectual property in the nanocrystals area. Baxter focuses mainly on the development of intravenous nanosuspensions. By now, no products are on the market, i.v. products are more complex to develop than oral products, e.g. due to the interaction with cells (cf. 4.1). A variation of this process is the counter flow precipitation. In this process, the solvent and non-solvent are mixed in two counter flows. Thus, the crystals precipitate at the interface [33].

The smartCrystal technology was developed by PharmaSol Berlin. Since 2007, this technology is owned by Abbott Lab. US and marketed by Soliqs as the drug delivery company of Abbott. It is not only one technology but a number of different combination processes which either accelerate production by reducing e.g. the number of passes through the homogenizer or lead to very small nanocrystals below 100 nm. Such small nanocrystals are difficult to access via pearl milling or "simple" high pressure homogenization, especially in large scale industrial production. The combination process H69 is a parallel flow precipitation and subsequent high pressure homogenization (HPH), whereas the precipitation takes place in the cavitation zone or just before the cavitation zone of the homogenizer (=cavi-precipitation) [34]. In the H42 process, spray-drying and HPH are combined [35], in H96 lyophilization and HPH [36] yielding nanocrystals ≪100 nm. The smartCrystal technology comprises different patent families, being a toolbox for tailor-making nanocrystals with optimized properties for different applications. For details of the process, it is again referred to [27].

4. Cellular interaction and intracellular delivery

4.1. General aspects of the fate of nanocrystals in the body / nanotoxicology

For many years, one looked uncritical at nanoparticles, and one has seen only the product advantages in many different fields. In the recent years, there is an increasing concern about nanotoxicity, especially because of the ability of nanoparticles to enter the cells, and causing damage to single cells, or even having systemic effects, e.g. by irritation of the immune system. Therefore, interaction with cells and uptake has to be considered when developing nanoparticle products.

The uptake of particles is a function of size. Injected pharmaceutical microparticle products (e.g. Parlodel) with a size in the range of about 50–100 μm cannot be taken up by cells at all, because the particles are larger than the cells of the body (rather 6–10 μm). Nanoparticles up to 1000 nm and particles with a size of a few micrometers can only be taken up by cells with phagocytic activity, e.g. the macrophages of the mononuclear phagocytic system (MPS). Particles can be phagocytosed by the macrophages e.g. in the liver, spleen and the lung, by cells of the Peyeŕs patches and by Langerhans cells in the skin. There are only a limited number of cells able to take up the particles. In addition, some of these cells are not easy to access. Consequently, the toxic risk is limited. This is different for particles with a size below 100 nm. These particles can be taken up by all cells by endocytosis. Therefore, they can be considered as high risk nanoparticles. However, at present, there is still no official international and general accepted definition about nanoparticles. Nevertheless, based on these considerations, some organizations define nanoparticles as particles with a size below 100 nm (e.g. BSI in the UK [37]) or the European Union in the latest cosmetic regulations [38]). In conclusion, the nanoparticles in the size range between 100 nm and <1000 nm and which are not taken

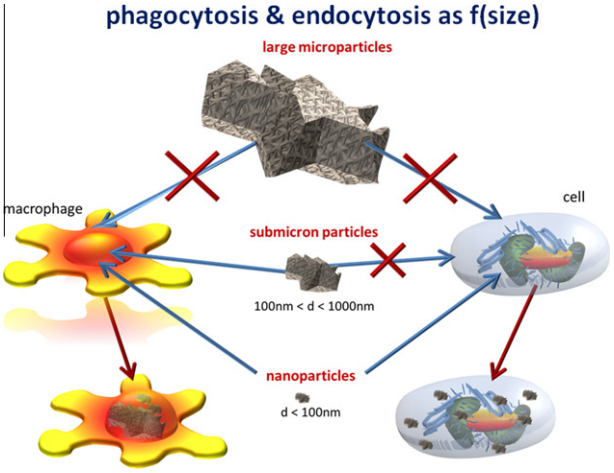

Fig. 3. Classes of particles and their interaction with cells as relevant parameter for potential nanotoxicity effects: large micrometer particles (>10 μm), submicron particles and a few μm, nanoparticles – defined as particles <100 nm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

up by endocytosis can be better classified as submicron particles. This will lead to a clear differentiation in size and toxic potential/tolerability. Fig. 3 shows the suggested new classes of particles and the related effects on cells.

Another important factor for the intracellular fate is the persistency of the nanoparticles in the body. Can they be degraded in the body or at least be eliminated from the body, or are they "biopersistent"? It is important that the particles can be degraded in the body and are not degraded by bacteria or cells in the environment, i.e. biodegradation in general is not sufficient. A nice example is polyhydroxybutyrate (PHB), a polymer investigated for production of polymeric nanoparticles [39,40]. It is a storage polymer in yeast cells, e.g. in *Bacillus megaterium* or *Ralstoniaeutrophus*, which is produced in response to physiological stress and used as an energy depot later on. Thus, the polymer and subsequently also polymeric particles of PHB can be degraded in these yeast cells, but not in the human body. Non-degradable nanoparticles can normally not be eliminated. They are too large for renal clearance. After cellular internalization, the particles stay within the cells and are normally not exocytosed anymore. The cells serve as waste deposit site for these nanoparticles. Fullerenes and carbon nanotubes (CNT) are

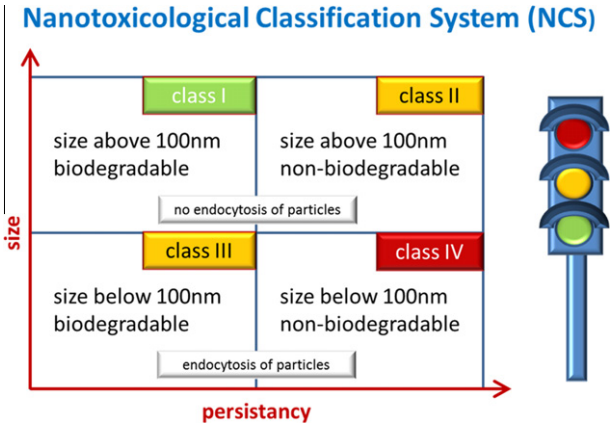

Fig. 4. Suggestion of a nanotoxicological classification system (NCS) for a precise differentiation of nanotoxicological risks derived from nanoparticulate carriers in the future. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

still proposed in the literature for targeted drug delivery or as parenteral controlled drug release devices. However, these modifications of carbon are not biodegradable; they stay forever in the body. Therefore, they have no chance for acceptance for pharmaceutical products by the regulatory drug authorities. Based on this, the particles can be again divided in a high risk class of the biopersistent nanoparticles and a low risk class of the degradable nanoparticles. By combining the size related risks with the risks derived from persistency, a nanotoxicological classification system (NCS), similar to the BCS after Amidon, is proposed in this article. Being based on the structure of the BCS system, it would allow a clear and straightforward justification of possible toxicological risks of particulate carrier systems. The suggested NCS is depicted in Fig. 4.

These size and persistency considerations have already affected the latest regulations of the EU for cosmetic nano products. Cosmetic products need to be labelled as nano products when they contain particles which are both, smaller than 100 nm and simultaneously biopersistent (corresponding to class IV of the NCS). Hence, if a product contains particles – such as the nanocrystals – smaller than 100 nm, but biodegradable (i.e. class III NCS), it is no declarable nano product.

The nanocrystals of pharmaceutical drugs or cosmetic actives are therefore a priori low risk or non-risk nanoparticles. However, even biodegradable nanoparticles can cause undesired systemic effects in the body or intracellularily until "the end of their existence", i.e. the complete dissolution in case of nanocrystals. An example is the irritation of the immune system when the particles are taken up by the respective cells of the immune system, triggering an immune response or irritation of the system. Therefore, such potential effects need to be carefully investigated when developing products, even with biodegradable nanoparticles.

4.2. In vitro interaction with cells/nanotoxicity aspects

There are very limited data about the in vitro tolerability of nanocrystals in cell cultures and also about the tolerability on certain organs, e.g. skin using artificial skin. The background is that mankind lives with nanoparticles of drugs or actives since its existence. Each drug microcrystal applied will convert to a nanocrystal during its dissolution towards the "end of its existence". No side effects are known from such naturally formed nanocrystals; therefore, the same good tolerability is assumed when applying nanocrystals directly themselves.

Nanocrystals of the novel, patent-protected secretory phospholipase A_2 inhibitors ($sPLA_2$) PX-13 and PX-18 [41] were produced by high pressure homogenization [42]. The particle size was in the range of 130–280 nm. Selective inhibitors of $sPLA_2$ are anti-inflammatory agents and considered as useful in the therapy of psoriasis, but also various other diseases related to phospholipase (PLA). The PX molecules are poorly soluble; therefore, they needed to be formulated as nanocrystals to make them biologically active.

The tolerability of PX-13 and PX-18 nanocrystals was evaluated in primary human fibroblasts and keratinocytes monolayer cell cultures using the MTT and the neutral red test (viability tests). The drug betamethasone was used as comparison. The measured effects are of course a superposition of the drug action and the nanocrystals as physical form, a differentiation is not possible. The cytotoxicity of the PX-13 and PX-18 nanocrystals was found similar or even below the one of betamethasone [43]. The dermal safety is therefore higher than for betamethasone, and the two nanocrystal formulations are promising candidates for a dermal product development.

Reconstructed human epidermis (EPISKIN) was used for the evaluation of the skin irritation potential of PX-13 and PX-18 nanosuspensions. By applying 5% nanosuspensions, no irritation was found [44]. For the evaluation of the eye tolerability of PX-13 and PX-18 nanosuspensions, the heńs egg-chorioallantoic membrane (HET-CAM test) was used. Both nanosuspensions did not show any toxic reaction, and they were classified as well tolerable [45]. These studies proved the dermal and ocular safety of the new phospholipase A_2 inhibitors PX-18 and PX-13, when formulated as nanosuspensions [46].

In vivo, even protective effects were found, which of course are not attributed to the nanocrystals itself, but the drug action of the PX molecules. Neuroprotective effects of PX-18 nanocrystals were observed in cerebral ischemia/reperfusion in gerbils [47,48]. PX-18 nanosuspension preserved the microvascular reactivity after cerebral ischemia in piglets [49]. Important is that no toxic effects in these in vivo studies were observed which could have been caused by the nanocrystals.

Dermal nanocrystals are on the market in cosmetic products, more precise submicron crystals because the size is larger than 100 nm. Rutin submicron crystals are in the line JUVEDICAL by Juvena Switzerland. Hesperidin crystals are in the product platinum rare by the company la prairie. It is one of the most expensive cosmetic products on the world. The crystal suspensions are distributed as concentrates by the company Dr. Rimpler GmbH in Wedemark nearby Hannover in Germany (www.rimpler.de). The suspension concentrates did undergo the safety tests for cosmetic products. In the HET-CAM test and in the human patch test, no irritation potential was found (product information sheets Dr. Rimpler GmbH). The dermal safety of the finished products was also tested by the manufacturers. In an in vivo study, the anti-oxidant effect of the nanocrystal formulations was proven [50]. Rutin nanocrystals proved about 1000-fold more active than the water soluble synthesized rutin-glycoside derivative [14,51]. Other safety-proven nanosuspension concentrates are e.g. hesperetin, apigenin and resveratrol.

Investigations of dermally applied solid lipid nanoparticles showed that the solid particles remained on the skin did not penetrate into the epidermis, but showed some location in the gaps around the hair follicles [52]. The same can be assumed for the nanocrystals; therefore, no direct interaction is expected with the living keratinocytes. The mode of dermal action of nanocrystals is explained via the increased saturation solubility, leading to an increased concentration gradient and subsequently increased penetration into the skin. In addition, lipophilic molecules penetrate better than very hydrophilic ones. This contributes to the superiority of the lipophilic rutin from the nanocrystals compared to the water soluble hydrophilic rutin-glucoside (Fig. 5).

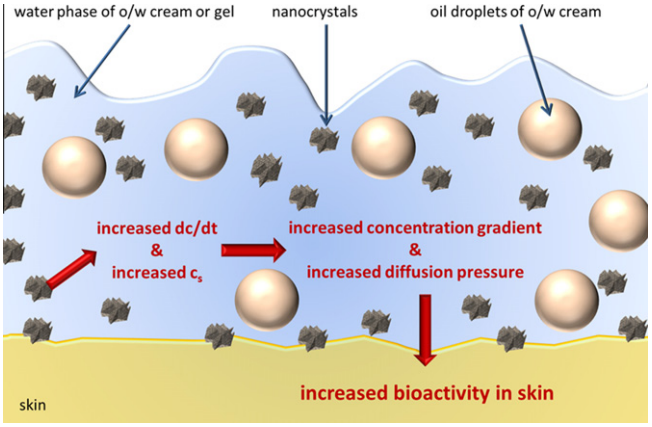

Fig. 5. Mechanism of action of nanocrystals applied to the skin in the water phase of a cream or gel (explanation cf. text). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4.3. Interactions after oral administration of nanocrystals

After oral administration, the nanocrystals are expected to primarily dissolve in the gastrointestinal tract. In studies with non-biodegradable model particles, it could be shown that the uptake of nanoparticles from the gastrointestinal tract is negligible [12,53–55]. There is limited uptake by the lymphatic system, e g. as assumed for testosterone nanocrystals [56]. Testosterone is heavily metabolized by the liver, and the concentration in the blood is explained by lymphatic absorption, e.g. from the product Andriol® Testocaps [57]. Lymphatic uptake of nanoparticles was reported for duodenally administered solid lipid nanoparticles (SLN) [58,59]. The SLN lead even to a very limited accumulation in the brain. Such effects are considered minor or not existing for nanocrystals, because such uptake processes are superimposed with the fast dissolution of the nanocrystals. Normally, they will be dissolved before entering such uptake pathways. Therefore, after oral administration in vivo, adhesion to the gut wall will take place as only cellular interaction, which is to be more precise primarily adhesion to the mucus layer. This adhesion process is pronounced and obviously very reproducible, explaining the reduction in the intra-subject and inter-subject variation of oral bioavailability [28,60].

4.4. Interaction with the MPS cells in vivo

The situation is completely different after intravenous (i.v.) injection. The MPS as "radar system" of the body normally detects the nanocrystals as particles being foreign to the body. Removal of foreign particles from the blood stream by phagocytosis by the MPS cells is very fast and efficient. Typically, within 5 min after injection, up to 90% of the injected dose is taken up by the liver macrophages (Kupffer cells) and about up to 5% by the spleen macrophages [61]. Typically, nanocrystals need 5–10 min for dissolution, depending on the particle size ($\gg$100 nm). As a consequence, non-dissolved nanocrystals are taken up to a large extent by the liver and to some degree by the spleen.

This uptake has several consequences or biological effects. A concentration of drug nanocrystals in the macrophages can lead to cytotoxic effects, especially when delivering anti-cancer drugs, i.e. chemotherapeutic agents. The uptake by the liver changes the pharmacokinetic profile of the drug compared to an injected solution of a poorly soluble drug (e.g. drug solubilized by surfactants). Therefore, it is very important to note that such a drug nanosuspension cannot serve as generic competitor product to an original solution product. After accumulation of the drug in the liver, the liver acts as depot slowly releasing the drug into the blood. Nevertheless, this can be a positive effect for some treatments. A nanosuspension injection could replace a longer lasting infusion of a drug solution.

Nevirapine nanosuspension for HIV therapy was injected i.v. and the organ distribution determined. The nanocrystal size was about 480 nm, as determined by photon correlation spectroscopy (PCS). As theoretically expected, 39.6% of the administered dose was found in the liver, 37.2% in the spleen, but only 7.9% in the heart and 11.5% in the kidneys [62,63]. Obviously, the nanocrystals dissolved too slowly to avoid recognition and uptake by the macrophages. There is no major accumulation in organs without phagocytic cells.

A paclitaxel nanosuspension was produced as alternative formulation to Taxol, to avoid Cremophor EL and related side effects. The nanosuspension was composed of 1.2% paclitaxel, 1.0% Phospholipon 90, 0.5% Poloxamer 188, 0.2% sodium cholate and water for injection up to 100%. The particle size was about 330 nm as determined by PCS [64]. Toxicity and treatment efficiency was studied in nude mice (NMRI nu/nu) with hetero transplanted mamma carcinoma MDA-MB 435 and MDA-MB 436 cell lines. By avoiding Cremophor EL, the lethal dose (LD50) was doubled for the nanosuspension. The injected maximal tolerated dose was 3×100 mg/kg for the nanosuspension compared to 3×40 mg/kg with Taxol. No signs of acute toxicity due to the liver accumulation were observed. Treatment efficiency in terms of tumor reduction was similar for both formulations, but with the nanosuspension a higher dose was required (3×40 mg/kg with Taxol (=maximal tolerated dose!) and 3×60 mg/kg for the nanosuspension, MDA-MB 436 model) [64]. This can be explained by the uptake in the macrophages of the liver, and this reduces the free concentration of the drug in the plasma compared to Taxol injected solution. The prolonged release from the liver had obviously no detectable positive effect on the tumor reduction in this study. Depending on the type of tumor and the drug used – at identical AUC – sometimes it is better to have a high plasma concentration for a short time (=bolus injection of a solution), sometimes to have a lower but longer lasting plasma concentration (=prolonged release from dissolving nanocrystal depot in the liver).

Itraconazole nanosuspension with a size of about 600 nm (laser diffraction) was produced and intravenously injected. The acute toxicity, pharmacokinetics and organ distribution were studied in rats, treatment efficiency in a rat model challenged with *C. albicans*. For comparison, a solution was injected, the commercial product SPORANOX® [65]. In this product, the itraconazole is solubilized by a cyclodextrin (HP-CD), observed nephrotoxicity is attributed to the HP-CD, not to the drug itself. The authors found changed pharmacokinetics with reduced c_{max} and an increased plasma half-life (15.6 h with the nanosuspension at a dose of 20 mg/kg, 5.05 h when given as SPORANOX®). The itraconazole concentrations were sustained much longer, explainable by the release from itraconazole nanocrystals taken up by the liver. Tissue concentrations were highest in liver and spleen, showing the highest values initially after injection. Then, the concentration in these organs declined which supports the proposed release of drug to the blood. The acute toxicity was reduced; this allowed – identical to paclitaxel nanosuspensions – higher doses. In this study also, a higher survival was found when treating with the nanosuspension. Based on the different pharmacokinetics, an itraconazole nanosuspension could not be placed on the market as a generic product to SPORANOX®; it would require a complete new registration process. The related costs need to be put into relation to the achievable annual sales, to decide if such a new development is financially viable.

Opsonins are blood plasma proteins that act as binding enhancers for particles to macrophages and thus trigger the phagocytotic uptake. Therefore, to avoid the interaction with the macrophages and particles, the adsorbance of opsonins from the blood onto the nanocrystal surface needs to be avoided. One has to create stealth nanocrystals analogous to the stealth liposomes [66,67]. The polyethylene glycol (PEG) chains on the particle surface reduce adsorption of proteins from the blood, important factors are density and length of the PEG chains [68]. Such in the blood circulating nanocrystals could dissolve within 5–10 min to yield a pharmacokinetic profile identical to a solution. To select the correct surface modification of the nanocrystals, the protein adsorption patterns from plasma and serum can be determined in vitro. The particles are incubated in plasma or serum, the excess removed, the adsorbed proteins are washed off from the particle surface and analyzed by two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) [69]. Adsorption of opsonins and activation of the complement system needs to be avoided [70].

Alternatively, nanocrystals can be produced with a size distinctly below 100 nm (e.g. smartCrystals). They dissolve in an instant after injection, because their saturation solubility increases exponentially with decreasing size, being most pronounced below

100 nm. In addition, they have a very large surface area. Both factors enhance the dissolution velocity [15], (cf. 2).

4.5. Interaction with the endothelial cells of the blood–brain barrier (BBB)

To direct nanocrystals to other cells in the body than the MPS, firstly they need to avoid recognition by the immune system to enable them to circulate in the blood. Secondly, a homing device needs to be attached to the particle surface mediating the attachment to the surface of the target cell and stimulating subsequent internalization. In addition, the travelling to the target cells needs to be fast, because during the travel the nanocrystal is continuously dissolving.

To create stealth nanoparticles, the surface of the nanocrystals can be modified by employing different surfactants and surfactant mixtures until surface properties are obtained which avoid adsorption of opsonins in the blood. The absence of opsonins can be checked in vitro by 2-D PAGE as described above. Examples for homing devices are antibodies, lectins, and sugars such as mannose with binding activity to the mannose receptor on cell membranes. However, very often the binding of the homing device to the particle surface leads again to recognition by the immune system and uptake by the MPS cells. A very smart approach is the targeting of cells by the concept of "differential protein adsorption", developed already in 1989 [71].

The concept is very simple. The surface properties of particles such as surface hydrophobicity, charge, presence and concentration of certain functional groups determine which proteins from the blood adsorb onto the particle surface after i.v. injection (=composition of the protein adsorption pattern). The protein adsorption pattern determines to which cells the particles attach, i.e. determines the organ distribution (Fig. 6). If this correlation is known, it can be controlled and exploited to design target cell specific nanocarriers. The particle properties are adjusted this way that the particles adsorb automatically preferentially this protein in the blood, which mediates the uptake by the target cells.

Kreuter at al. observed by chance that drug-loaded polymeric nanoparticles stabilized with Tween 80 on the surface could transport the drug dalargin to the brain. Nanoparticles with no Tween 80 on the surface (=different surface properties) did not (=negative control) [72]. Analyzing the protein adsorption patterns on these two nanoparticles revealed that apolipoprotein E (Apo E) was preferentially adsorbed on the surface of the brain-specific nanoparticles [73,74]. Thus, it was assumed that it was the targeting mediating moiety to the endothelial cells of the blood–brain barrier (BBB). To prove this, Apo E was attached to the surface of the negative control. Now also these nanoparticles transported dalargin to the brain [75,76]. The same targeting to the brain with Tween 80 stabilized nanoparticles could be achieved using other drugs, e.g. paclitaxel [76], doxorubicin [77] and tubocurarine [78].

The Tween 80 surface of the nanoparticles possessed the "right" properties that Apo E was preferentially adsorbed, and it was not detectable at all on the surface of the negative control. Apo E attached the particles to the apolipoprotein E receptor of the endothelial cells of the BBB; subsequently, the particles are taken up by endocytosis into the cells. In the cells, the drug is released from the particle and diffuses into the surrounding brain tissue (Fig. 7), as shown by Kreuter for doxorubicin [79]. The same principle was transferred to nanocrystals. Atovaquone nanocrystals were stabilized with Tween 80. In vitro, it could be shown by 2-D PAGE that Apo E adsorbed onto these particles from plasma. They were intravenously injected to treat toxoplasmosis, the parasites could be efficiently eradicated in the brain [80,81]. The nanocrystal size

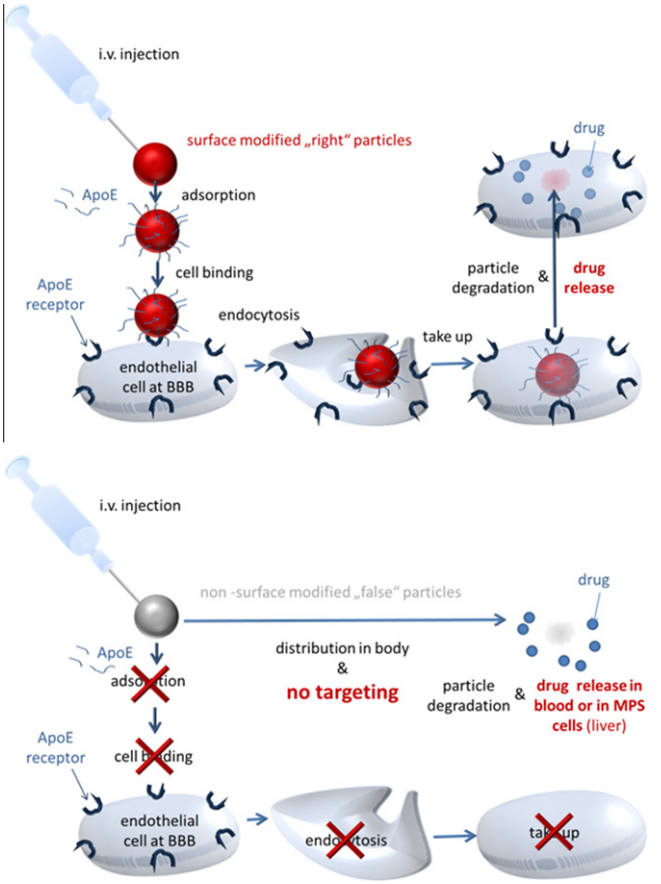

Fig. 7. Principle of Apo E mediated uptake by the endothelial cells of the brain: after i.v. injection. Upper: the nanoparticles with "right" surface properties adsorb preferentially Apo E. This mediates binding to the Apo E receptor on cell membranes, the binding triggers endocytosis, and drug is released from the nanoparticles and diffuses into surrounding tissue. The nanoparticle itself is degraded in the endosome/cytoplasm. Lower: the nanoparticles with "false" surface properties do not adsorb Apo E. Therefore, no cell targeting is observed. The drug is released either in the blood or in the MPS cells of the liver. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

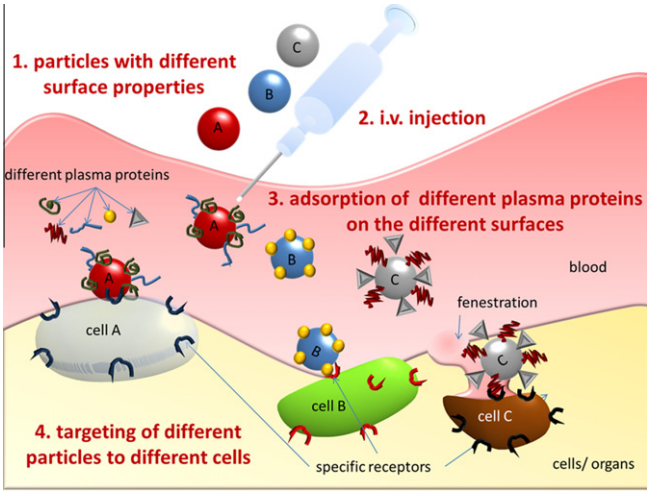

Fig. 6. Principle of "differential protein adsorption": The adsorbed proteins in the blood differentiate to which cells the nanoparticles are going after i.v. injection. The cells need to be accessible from the blood, e.g. endothelial cells or cells via accessible fenestrations. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

was 279 nm, measured by PCS. This is a size too large for endocytosis. However, on their travel to the BBB, the nanocrystals started dissolving, shrinked in size and might have had a sufficiently small size when arriving at the BBB.

The targeting to the BBB is presently being exploited for the development of brain-specific pharmaceutical products (e.g. company Capsulution in Berlin/Germany). The major obstacle is the competitive uptake of the polymeric nanoparticles by the MPS cells by phagocytosis and the endothelial cells of the BBB. There are different data in the literature about the percentage of particles reaching the brain. It can be summarized that it is about or even less than 1% of the injected dose arriving at the brain. This creates problems in reaching a therapeutic drug concentration in the brain. This problem is even more pronounced when using polymeric nanoparticles because the drug loading is relatively low (e.g. dalargin was only bound to the surface of the nanoparticles). Here, nanocrystals have a clear advantage, they consist of pure drug, and the drug loading is 100% (cf. Fig. 1). In addition, by modifying the surface properties to reduce opsonin adsorption, a higher percentage of nanoparticles can be shifted to the brain. This is a realistic assessment, because even with the non-surface optimized atovaquone nanocrystals efficient killing of parasites in the brain was achieved [80].

5. Conclusions and perspectives

Besides the liposomes, the nanocrystals are the most successful nanocarrier when considering the short time between invention and first marketed products, the total number of products on the market and in clinical phases, and also having with Tricor the first nano block buster product (US sales > 1 billion US $ per year). In terms of interaction with cells, they belong to the low risk class of nanoparticles, because the size can be made >100 nm, and they are biodegradable (they just dissolve in presence of sufficient water). The nanotoxicity profile seems to be uncritical.

In dermal and oral administration, the nanocrystals adhere to cells, intracellular uptake plays no/little role. In contrast to this, intracellular uptake plays a key role after intravenous injection. Uptake by the MPS cells (e.g. liver) can optimize blood profiles in treatment (e.g. sustained or prolonged release), but also cause cytotoxic effect in case of too high nanocrystal concentrations in the macrophages. The balance between uptake by MPS cells and target cells is critical for a successful therapy. The different cell uptakes can be modulated by surface modification of the nanocrystals, affecting the protein adsorption pattern as determining parameter for the cell affinity. However, by now, very little research has been done in this field. Obviously, the empirical approach is taken: preparing nanosuspensions with different stabilizers and looking directly at the organ distribution in animals to identify a suitable composition (trial and error approach, no controlled development).

Too little studies have been performed by now looking at the detailed intracellular fate of the nanocrystals. Of course, an obstacle is that the nanocrystals undergo continuous dissolution during her travel inside the cells. Basically, they should follow the same pathway as similarly sized non-biodegradable nanoparticles (e.g. polystyrene) after phagocytosis or endocytosis – but this need to be proven. To get more knowledge about the intracellular fate might open new treatment approaches.

There is also a lack of cytotoxicity studies. Because the nanocrystals dissolve, and each applied drug micrometer particle is at the end of its dissolution a nanocrystal, they are considered as harmless. This classification appears justified because there are no contradicting reports. The limited cytotoxicity studies done are often very crude by looking simply at the viability. It would make sense to look at the molecular level, e.g. LDH release, loss of potassium from inside the cells, and at the cytokine production as done for the solid lipid nanoparticles [82]. This revealed clear differences between different biodegradable lipids. Such investigations are essential for the better understanding of nanocrystals on the cellular level and can also open new nanocrystal applications.

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