Nanocrystals: Production, Cellular Drug Delivery, Current and Future Products

Rainer H. Müller, Ranjita Shegokar, Sven Gohla, and Cornelia M. Keck

Abstract Drug nanocrystals are a formulation principle for systemic and also intracellular delivery of poorly soluble drugs. Their production by bottom up techiques (precipitation – hydrosols, Nanomorph) and by top down techniques (bead milling – NanoCrystal®, high pressure homogenization – DissoCubes®, NANOEDGE®) is briefly described, representing the first generation of nanocrystals. The second generation, the smartCrystal®, is produced by combination processes. They are featured by e.g. increased physical stability and/or smaller sizes (<100 nm), favourable when exposed to the destabilizing electrolytes in biological fluids and for uptake by cells by pinocytosis. The lab scale processes were successfully transferred to industrial scale by using discontinuous bead mills and high capacity homogenizers (top down), precipitation can be performed by static blenders. According to the nanotoxicological classification system (NCS), the nanocrystals belong to class I, being highly tolerable. They can be produced using only regulatorily accepted excipients. Both ease the way to the patient and market. Nanotoxicity studies confirm the good tolerability. The nanocrystal products on the market are no direct intracellular delivery systems. They transport drug to the biological barrier and then promote penetration and permeation of drugs in molecular form through barriers and cellular membranes (cellular delivery mechanism I). Formulations based on the cellular uptake of nanocrystals are still in development (cellular delivery mechanism II). Examples are i.v. targeting to endothelial cells of the blood-brain barrier and the loading of blood cells (monocytes, erythrocytes) to use these cells as transport vehicles for the nanocrystals. By now, very little work has been done to study and actively modulate the intracellular fate of nanocrystals.

Keywords Nanocrystals • Saturation solubility • Dissolution velocity • Dermal delivery • Oral delivery • Intravenous delivery • Nanotoxicity

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1 Introduction

Soluble drugs can reach their target in the body (organ, cell, cellular compartment) by a simple diffusion process of the drug molecules in the body fluids. In contrast to this, poorly soluble drugs – due to their insolubility in body fluids – need to have a carrier which carries them to the target, e.g. particulate nanocarriers. Diffusion in the body fluids is a non-specific, i.e. non-directed process. Soluble drug molecules distribute regarding distribution velocity and organ pattern according to their molecular properties (e.g. molecular weight, diffusion coefficient, log P value, permeability of membranes for the specific molecule, clearance). In contrast to this, incorporation of a molecule in a nanocarrier allows to target it to specific sites in the body, certain organs, specific cells within this organ or ideally to a specific cell compartment.

Such nanocarriers are e.g. micelles, polymeric nanoparticles or lipidic systems such as nanoemulsions, liposomes, transfersomes, niosomes or lipid nanoparticles made from solid lipids (e.g. solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) (Müller, Shegokar, and Keck, in press). A basic problem of these nanocarriers is that very often the loading capacity for drug is relatively low, especially when drugs are just bound to the surface of a nanocarrier (e.g. dalargin to the surface of polymeric nanoparticles (Kreuter et al. 1997)). This is a very pronounced problem when the aim is to target. Only a limited fraction of the administered carriers reach the target (assumed 10%). If the loading capacity is low (e.g. 10%), only a small fraction of the totally administered drug reaches the organ which might not be enough to reach a therapeutic level for cure of the disease. Therefore a nanocarrier would be ideal having a loading capacity of close to 100%.

This is realized by the nanocrystals. They are particles consisting of drug only without any matrix material as e.g. in polymeric nanoparticles (polymers) or liposomes (phospholipids). They are only stabilized by an adsorbed surfactant layer or sterically stabilizing polymer layer. Such adsorption layers on nanoparticles are typically 2–5 nm, maximum about 10 nm in thickness. Considering cubic nanocrystals of 500 nm size and the adsorption layer as part of the nanocrystal, this corresponds to maximum just 6% of the volume. Practically the nanocrystals can simplified be considered as nanocarriers with 100% loading capacity.

Major prerequisites for use of a nanocarrier in therapy are the possibility of controlled production, ability to produce on large industrial scale, accordance with regulatory requirements (e.g. status of excipients), ability for efficient delivery of drugs, ideally target to the interior of cells, and in case of nanocarriers the increasing need of data to prove the absence of nanotoxicity. Ideally the nanocarriers used should belong to class I of the nanotoxicological classification system (Müller, Gohla, and Keck, in press). These issues will be presented and discussed within this book chapter.

2 Definitions and Special Properties

Drug nanocrystals are particles consisting of pure drug only, and being by definition in the nano size range, i.e. below 1,000 nm to a few nm. They are in the crystalline state, due to this they are of cuboid shape. Trade names are NanoCrystal® (élan, prev. Nanosystems), DissoCubes® (SkyePharma, prev. PharmaSol) and smartCrystal® (Soliqs/Abbott, prev. PharmaSol). Drug nanoparticles can also be amorphous. In this case, in a strict sense they should not be called nanocrystals, trade name is Nanomorph (Soliqs/Abbott). Because of the amorphous state and lack of ordered, periodically repeating structure, they are spherical (Fig. 1).

There are different views how big a nanoparticle is. The above definition is based on dimensional considerations, i.e. the complete nanometer range, and typically used by most pharmacists. The US Food and Drug Administration (FDA), the National Nanotechnology Initiative (NNI) and the new European Cosmetic regulations consider nanoparticles as particles below 100 nm (Regulation (EC) No 1223/2009; National Nanotechnology Initiative (NNI) 2001). This can be rationalized by the increased toxicity risk when going below this size threshold (cf. 5, NCS classes). Some colloid scientists consider a "real" nanoparticle below about 20 nm.

In case the nanocrystals or amorphous drug nanoparticles are dispersed in a liquid, this is called "nanosuspension". Nanocrystals can be dispersed in either water or in non-aqueous dispersion media. Examples for non-aqueous media are oils, paraffins, liquid polyethylene glycols (PEGs) but also solid PEGs. In the latter case it is a solid nanosuspension or solid nanodispersion, analogous to classical solid dispersions of particles or solid dispersions of solutes.

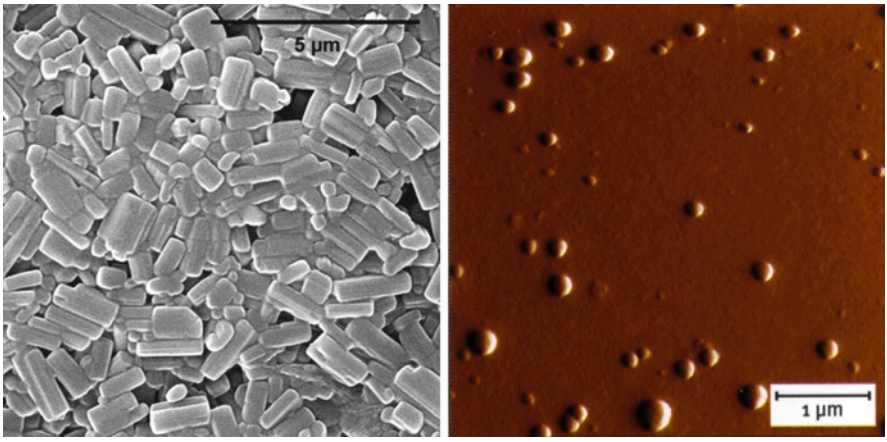

Fig. 1 Crystalline nanocrystals with cuboid shape (*left*, Courtesy by Böhm (1999), Modified) and nanoMorph amorphous drug nanoparticles of spherical shape due to lack of ordered crystal structure (*right*, Courtesy by Soliqs, Ludwigshafen, Germany)

Special properties of nanocrystals are an increased saturation solubility c_s and an increased surface area A, both leading to an increased dissolution velocity dc/dt according to the Noyes-Whitney equation (Buckton and Beezer 1992). The increase in saturation solubility leads to increased concentration gradients at biological barriers and membranes (e.g. gut wall, skin, barriers such as blood-brain barrier), and subsequently to increased penetration into or permeation across (Keck and Müller 2010). The increased dissolution velocity has advantages, but implies the problem that the nanocrystals might be dissolved before reaching the cellular target. How to deal with this problem is discussed below. Another important feature is the adhesiveness to surfaces. Identical to any other nanomaterial, nanocrystals stick to surfaces due to increased interaction of their large surface with substrates. This adhesion process is very reproducible, and the reason for better pharmacological performance, e.g. minimized variation in bioavailability when using nanocrystals as delivery system (Liversidge and Conzentino 1995; Liversidge and Cundy 1995). The physical background of the nanocrystal properties is described in detail in (Müller et al. 2003).

3 Process Technology

3.1 Bottom Up Processes

In a bottom up process, one starts from a small unit and in the process the size is increased. In case of nanocrystals one starts from a molecular solution of the drug, the molecules are aggregated to form particles in the nanometer size range. Practically in most of these processes we have a classical precipitation. A solvent containing the drug is added to a non-solvent. The solvent can be water, or organic solvents, the non solvent can be fluids miscible with the solvent (e.g. ethanol, acetone) or even supercritical fluids, typically carbon dioxide.

The so called hydrosols by Sucker are generated by classical precipitation, they are crystalline (List and Sucker 1988). Applying special precipitation conditions leads to amorphous nanoparticles, a process developed by Auweter and co-workers at BASF Germany. Food products on the market based on this technology are carotenoid powders (Auweter et al. 2002). In the pharmaceutical area, amorphous precipitation is performed by Soliqs for its product NanoMorph. There is quite a variety of other precipitation methods described in the literature, e.g. high-gravity controlled precipitation technology (Chen et al. 2009) and flash nanoprecipitation (Bénet et al. 2002; Johnson et al. 2006). Also a number of supercritical fluid processes is applicable, which would actually require a review on their own. Therefore it is referred to (Byrappa et al. 2008).

Controlled precipitation is a little bit tricky to run, costly because solvents might need to be removed, solvent residues need to be controlled, with regard to many aspects more complex than some top down processes. This is the reason why there are no currently marketed pharmaceutical products for therapy made with this technology.

However, this should not lead to the conclusion that bottom up processes are not industrially viable! On the contrary, they might be the basis for the third generation of drug nanocrystals/amorphous drug nanoparticles. By precipitation very small sizes (<<100 nm) are accessible, which are not or only very difficult to access by top down technologies. They are of high interest especially for intracellular delivery because they can be internalized by endocytosis/pinocytosis, that means by many cells in the body and not only by macrophages.

3.2 Top Down Processes – First Generation Nanocrystals

In the top down processes one starts from a larger size unit and reduces the size to the nanometer range. Typically wet milling processes are applied. They can be differentiated in low energy milling and high energy milling processes, i.e. bead (pearl, ball) milling and high pressure homogenization. They are used for production of nanocrystals which can be referred to as the first generation: NanoCrystal®, DissoCubes® and NANOEDGE®.

The NanoCrystals® are produced by bead milling (Liversidge et al. 1992). A macrosuspension of the drug powder is fed to a bead mill, containing small hard milling beads (e.g. 0.2–0.4 mm diameter, made from zirconium oxide or hard polystyrene). The beads are moved by an agitator or by rotating the milling chamber itself, and the drug crystals are ground between the moving beads (Merisko-Liversidge 2002). The system can be discontinuous (mill filled with complete batch), or continuous by pumping macrosuspension through the mill. The lab scale milling process typically lasts a few hours, relatively low energy is applied for a longer time.

In the high energy process of homogenization (DissoCubes® (Müller et al. 1999)) the macrosuspension is passed through the homogenizer (piston-gap, jet stream) at pressure of 1,500 bar and higher. Typically 10–20 passes through the homogenizer need to be applied. In the first generation, the dispersion medium of the macrosuspension was water, cavitation was considered as major cause for size reduction. In the NANOEDGE® process, a pre-step of precipitation is performed, then the precipitated suspension is exposed to a high energy step, typically high pressure homogenization (Kipp et al. 2003). In a further development, precipitation can be performed in a counter flow process (Kipp et al. 2005).

3.3 Top Down Processes – Second Generation Nanocrystals

In further developments, the nanocrystal production was improved regarding:

- running of process (e.g. less homogenization cycles, production of final dosage forms)
- smaller particle sizes (<100 nm)
- physical properties of nanosuspension (improved physical stability, long-term and against electrolytes).

This was achieved by using combination processes, but also varying the dispersion medium. Water was replaced by non-aqueous dispersion media, or water mixtures (Nanopure®, Müller et al. (2000)). Oily nanosuspensions can directly be filled into capsules for oral delivery. Combination processes optimize in a first step the drug material to be more fragile in the second step of high pressure homogenization, or to yield more physically stable nanocrystals. Combinations are:

H42: spray-drying of drug solution and subsequent homogenization (Möschwitzer 2005)
H69: precipitation of suspension just before entering or in the cavitation zone of the homogenizer (Müller and Möschwitzer 2007)
H96: lyophilisation of drug solution and subsequent homogenization (Möschwitzer and Lemke 2007)
CT: bead milling followed by homogenization (Petersen 2006)

These different technologies are unified under the trade name smartCrystal® by Soliqs/Germany, offered as a toolbox of different processes for tailor-making of nanocrystals.

4 Process Scale Up

Lack of scale up ability is one of the reasons for the failure of interesting nanocarriers – feasible on lab scale – to enter the market. First of all, the process itself needs to be scaleable, secondly the process needs to be able to be qualified and validated, the production lines have to be regulatorily acceptable, and of course cost-effective (sufficient capacity per hour, limited manpower required for process, yield, costs of excipients). The costs of the production line are less critical, because it is a unique investment. Below the processes are discussed possessing from our point of view presently highest commercial potential, being already used in products or being closest to products. Therefore the supercritical processes are not considered.

4.1 Precipitation

Precipitation itself is basically a simple process when the precipitation conditions are established. Critical can be the scale up, because precipitation velocity is quite different on lab scale (e.g. 1 L volume) or on large scale in a 1,000 L container. Mixing of solvent and non-solvent is much faster in a small volume. The Ostwald-Mier region is passed very fast, which yields small crystals. A major development step, easing the scale up tremendously, is the static blenders. Precipitation takes place within the small volume of the static blender, being of similar size than the lab scale beaker. Lab scale conditions are imitated on large scale production.

Solvent and non-solvent are pumped via separate tubes into the static blender. The blender contains buffles leading to fast blending and precipitation. The system can be run continuously from two supply containers (Fig. 2, upper).

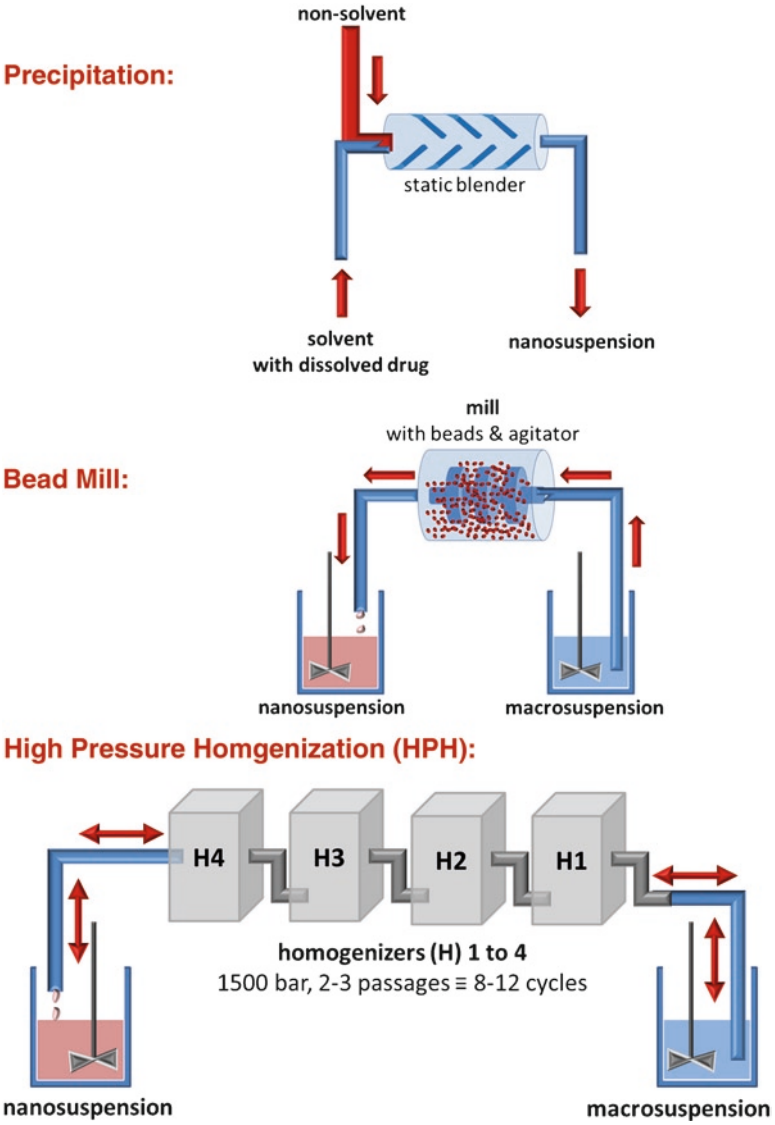

Fig. 2 Large scale production of nanocrystals (explanations cf. text): *upper* – solvent with drug and non solvent are mixed in a static blender; *middle* – macrosuspension is passed multiply from two containers through a bead mill; *lower* – four homogenizers in line for nanocrystal production, one pass is equivalent to four homogenization cycles

4.2 Bead Milling

Scale up of the bead mill in a discontinuous production is only feasible to a limited extend, because the weight of the beads (density zirconium oxide: approx. 6 g/mL (Zirconium dioxide – Wikipedia)) would lead to a too high weight of the mill. It needs to be also considered that about 70% of the mill volume is filled by beads, leaving only about 30% volume for adding the macrosuspension. A relatively high mill weight results compared to a low volume of suspension for processing. Therefore the continuous process is applied in pharmaceutical industry. The suspension is passed multiply through the mill (Fig. 2, middle). Very often, with just one passage a mean diameter in the nanometer range is obtained. The number of passages depends on the product requirements. About three to four passages are typically required to obtain a mean diameter below 500 nm. To minimize the content of crystals >1 μm (e.g. for i.v. injectables) about six to ten passages are needed. Typically after 10 passages, latest 15 passages very often the maximum dispersivity is reached. The perfectness of the crystals increases with decreasing size because the crystals break preferentially at their imperfections. At maximum dispersivity the energy is not sufficient to break these more perfect crystals. Therefore additional passages do not lead to further size reduction. On the contrary additional energy input can promote aggregation because the energy put in cannot be used any more for size reduction. This energy is now available to overcome the repulsive forces between crystals and to aggregate them.

4.3 High Pressure Homogenization

Homogenizers with a homogenization capacity of 1,000 L/h and more are commercially available. This is very favourable for large scale production and can compensate the need of 10 or even 20 passages through the homogenizer (= homogenization cycles) for the product. Compared to other equipment, the homogenizers are relatively low cost. To accelerate the process, four homogenizers can be placed in line, one passage through this line is equivalent to four homogenization cycles. The pumping velocity of the four homogenizers is electronically coordinated (Fig. 2, lower). Assuming a batch size of about half a ton, roughly 500 L, and ten production cycles on a homogenizer line with 1,000 L/h per homogenizer, one passage through this line is equivalent to four cycles and takes 30 min homogenization time. For the equivalence of ten cycles 1 h and 15 min are required, plus preparation and cleaning time.

Important for the output is also the solid concentration of the suspension to be processed. For both bead milling and homogenization concentrations in the range 10–20% are in most cases processable without sincere problems. With the bead mill sometimes concentrations up to about 40% are processable (depending on particle size of the starting material, affecting the viscosity). However, more

viscous suspensions are more difficult to process. Therefore at the end of the day it might be faster to have longer milling time but using a less concentrated, less viscous suspension.

5 Nanotoxicity and Regulatory Aspects

A key issue in nanocarrier development since decades is the regulatory status of the excipients used. In addition, in recent years potential nanotoxicty is coming increasingly into the focus of the consumer, triggered by often uncritical, simplifying reports in the popular press. Nanotoxocity is sincerely an issue, because by transferring material to the nanodimension its physico-chemical properties and subsequently its interaction with the biological environment, mainly the cells, change.

Many nanocarriers originating from the lab are made from materials which are not accepted for use by the authorities, e.g. newly synthesized polymers to give the nanoparticles a special performance. Industry is very reluctant to invest in expensive toxicity studies, especially when the result is questionable with a new compound, thus hindering these nanocarriers to enter clinical trials and the market. The situation is not that much different, when excipients are used which are accepted by authorities, but not accepted for the respective purpose. The classical example is the poly lactic glycolic acid polymer (PLG). They are on the pharmaceutical market as microparticles for injection (e.g. Enantone Depot, Decapeptyl Depot). However, when making small particles from this polymer, pronounced cytotoxicty was found in macrophage cultures (Smith and Hunneyball 1986). In contrast to 50–100 μm microparticles, particles with a size of a few μm and nanoparticles can be taken up by macrophages. Intracellular degradation leads to release of lactic and glycolic acids, causing at too high concentration the observed cytotoxicity – despite being both physiological compounds in the body.

In contrast to many other nanocarriers, the drug nanocrystals can be made from regulatorily accepted excipients only. They consist just of the drug and stabilizers. One can choose from a broad range of stabilizers regulatorily accepted in formulations for the respective administration route (dermal, oral, intravenous (i.v.), intramuscular, etc.). Examples range from various polymers for oral administration (e.g. polyvinyl alcohol (PVA), polyvinyl pyrrolidon (PVP)) including the basically membrane-toxic sodium dodecyl sulphate (SDS) to i.v. accepted lecithins, Tween 80, Poloxamer 188, low molecular weight PVP. This gives a big advantage to this delivery system regarding use in the clinic and entering the market. Of course, the question of potential nanotoxicity still needs to be discussed.

According to the nanotoxicological classification system (NCS, Fig. 3) different levels of tolerability and toxicity are distinguished, based on the particles size and the degree of biodegradability. There are two size classes of the NCS, nanoparticles in the range of about 100–1,000 nm can only be taken up by macrophages, possess therefore only access to a limited number of body cells, having therefore a lower toxicological risk. Nanoparticles below 100 nm can enter any cell of the body by

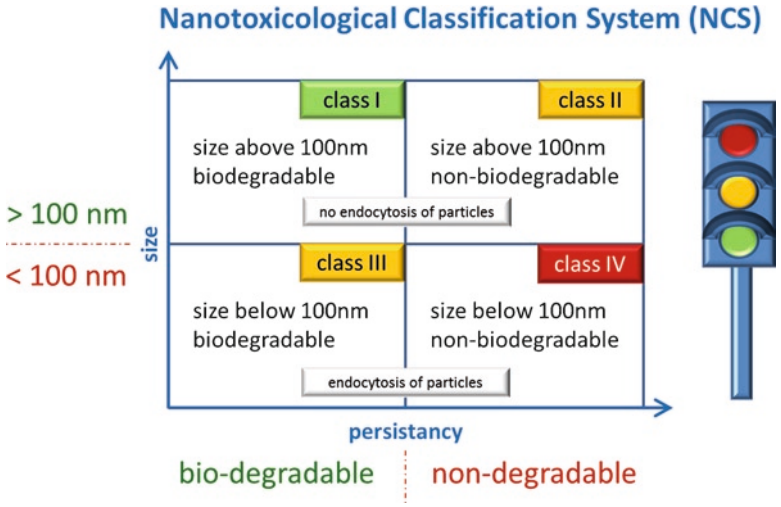

Fig. 3 The nanoparticles are differentiated in class I–IV with increasing toxicological risk, based on size (<100 nm, 100–1,000 nm) and biodegradability/non-biodegradability (i.e. persistency in the body) (Modified after Müller, Gohla, and Keck, submitted)

endocytosis/pinocytosis, having therefore a higher potential risk. Nanoparticles which are biodegradable in the body will disappear after some time, thus potential undesired effects are often limited to the time of existence of the particles. Biodegradable nanoparticles are therefore also a lower risk class. Non-biodegradable particles such as e.g. fullerens and carbon nanotubes (CNT) – often discussed as potential drug delivery systems – will stay forever. Non-biodegradable particles can cause continuing irritations, thus being excluded for the use as drug delivery system. Based on these considerations, the nanocrystals are in the lowest risk class I, or in class III depending on the size above or below 100 nm.

It should be kept in mind that also a biodegradable nanoparticle can cause toxic effects. For example during their life time biodegradable nanoparticles can be taken up by cells of the immune system and can cause irritation/activation of the immune system. Therefore also in this case assessment of potential nanotoxicity risks is meaningful. Assumingly because of the a priori assumed lack of nanotoxicty of the nanocrystals, there are very few reports about cytotoxicity investigations. Oral nanocrystal products are on the pharmaceutical market since 10 years, but no systematic investigations are published. Good tolerability can be assumed because mankind lives for centuries with drug nanocrystals present in the gastrointestinal tract (GIT). Each drug crystal orally administered will reduce in size during its dissolution, to a few μm and finally to the nanometer range prior to its complete dissolution. Nanosuspensions were mainly investigated regarding treatment efficiency, in vitro and in vivo, less looking at toxic effects. For example, the efficacy of atovaquone nanosuspensions was investigated against toxoplasma gondii in vitro

(Schöler et al. 2000) and in vivo (Schöler et al. 2001). Recently toxicity investigations were published on the new phospholipase A_2 inhibitors PX-13 and PX-18 nanocrystals (Pardeike and Müller 2010). The nanocrystals were invesitgated using the EPISKIN Test and the HET-CAM test to study the eye irritation potential. The nanosuspensions were found to be dermally safe, they were not or only slightly irritant to the eye.

It was also found that nanocrystals can even reduce irritancy to cell layers, e.g. the gastric wall. Nanocrystals of naproxen were shown to decrease the gastric irritancy (Liversidge and Conzentino 1995) compared to the drug powder. Intraperitoneally injected azodicarbonamide (ADA) was not irritant as nanosuspension, much better tolerated than irritant micrometer crystals (unpublished data). The underlying mechanism could be that the drug is more evenly, finely distributed about the walls of the gut or the peritoneum, similar to the improved tolerability of pellets compared to tablets loaded with irritating drugs. In addition, size and form of the drug crystals might also affect the tolerability (e.g. long needles compared to small cubes, e.g. similar to the toxicity of needle-like carbon nanotubes).

As a general aspect, nanocrystals normally change the pharmacokinetic profile of actives (i.e. higher c_{max}, shorter t_{max}). This can lead locally to a higher drug exposure of cells (e.g. kidney at higher plasma concentrations). This effect is well described for the nephrotoxicity of Amphotericin B. The nephrotoxicity is higher in formulations leading to higher concentrations of free Amphotericin B in the blood (Amphotericin B injectable solution versus Ambisome® liposomes). Definitely, there is a need for further closer examination of cellular effects of nanocrystals.

6 Nanocrystal Products on the Market – "Cellular Delivery Mechanism I"

6.1 Cosmetic/Dermal Market

Normally the cosmetic market watches carefully developments in pharmaceutical labs and industry to identify technologies and carrier systems with potential use in cosmetics. The classical example are the liposomes which appeared first on the cosmetic market (1986, product Capture by Dior) before entering the pharmaceutical market around 1990. In case of the nanocrystals this was different. The nanocrystals appeared first on the pharmaceutical market in 2000 (product RAPAMUNE® company Wyeth). The potential of nanocrystals for dermal delivery was realized a few years later (Petersen 2006). The first products were placed on the market in 2007 (line Juvedical, company Juvena).

In these products the delivery of the cosmetic active is not achieved by intracellular uptake of the nanocrystals. Based on the physical properties of the nanocrystals, they deliver molecules to the cell which dissolve from their surface, which we call delivery mechanism I. In delivery mechanism II, delivery of active to the cell takes

place by uptake of the nanocrystals themselves (cf. 7). Delivery mechanism I consists of the steps (Fig. 4, upper):

1. Formation of a supersaturated solution around the crystal, thus
2. creating a high concentration gradient between nanocrystal and target cell, and
3. fast replacement of diffused molecules by very fast continuing dissolution from the large nanocrystal surface (= depot).

Supersaturated solution, concentration gradient and dissolution are much faster compared to micrometer crystals, thus explaining the superior delivery properties

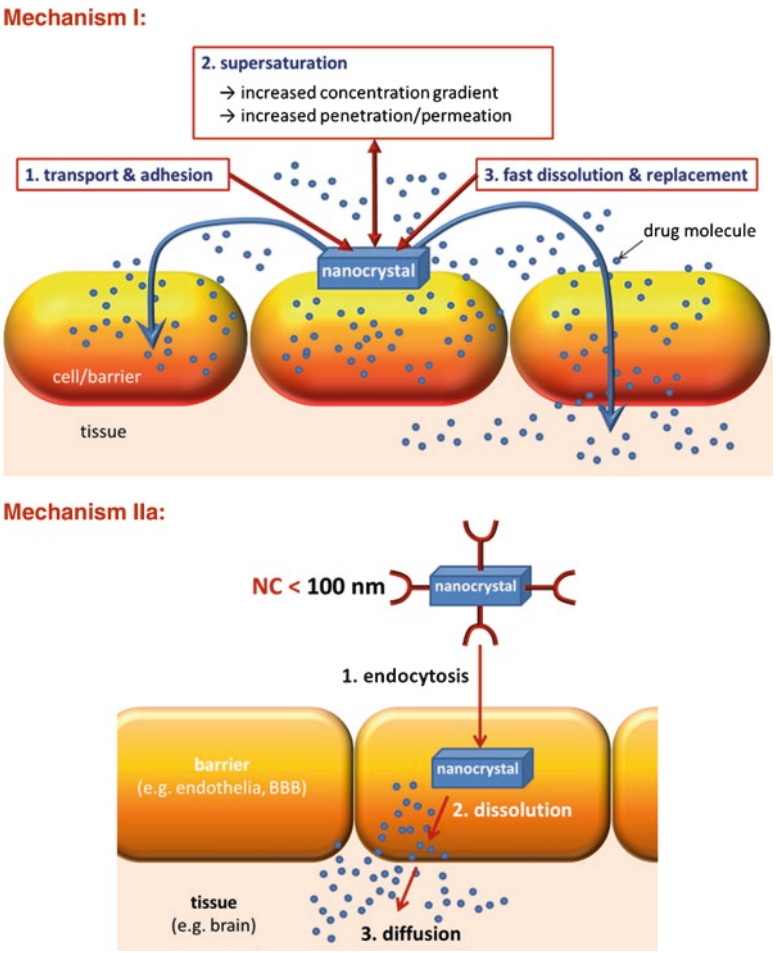

Fig. 4 Delivery mechanisms of drugs to cells/barriers via nanocrystals: mechanism I (*upper*) – the nanocrystals transport the drug to the cell, provide higher concentration gradient and the molecules diffuse into the membrane/cell. Mechanism II – the nanocrystals <100 nm enter the cell, delivering the drug to cell compartments or the drug diffuses from these cells to underlaying tissue (mechanism IIa, *middle*, e.g. endothelial cells of blood-brain barrier (BBB)), or the nanocrystals enter the cell and use the cell as carrier to their final target (mechanism IIb, *lower*)

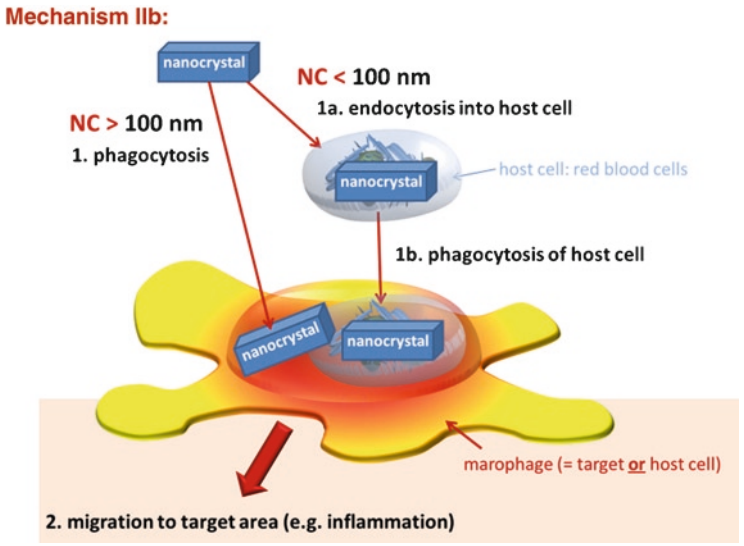

Fig. 4 (continued)

of nanocrystals. The mechanism will be explained in detail using the example of rutin and hesperidin (Petersen 2006).

Delivery of actives to the skin or any other target site can be studied by measuring concentrations at the target site or ideally by measuring the resulting effect. Measuring the effect assesses the overall performance of a delivery formulation, especially being essential when comparing two molecule derivatives (e.g. original molecule and derivative, e.g. glucoside). Rutin and hesperidin are poorly soluble antioxidants, the solubility is so low that incorporation as normal micrometer powder into a cream will lead to no effect. Therefore in the study by Petersen soluble rutin was made by glucosidation. The rutin-glucoside was compared in its antioxidative capacity to nanocrystals of the original poorly soluble rutin and in addition to hesperidin (Petersen 2006). In addition a formulation with alpha tocopherol acetate was run, which should make the skin more sensitive. To quantify the activity in the skin (= effect), the sun protection factor (SPF) was determined in humans under UV exposure. Antioxidant activity increases the SPF. Measurement of penetrated concentrations (e.g. tesa stripping test, biopsy) would have been less conclusive, because it does not consider potential different activities of the molecules in the cell (original versus glucoside). The overall performance of a formulation is a function of delivery efficiency and activity of the delivered type of molecule.

The rutin glucoside was dissolved at a concentration of 5.0% in the dermal test formulation. The nanocrystal formulation contained a depot of nanocrystals and a dissolved concentration of rutin of about 0.01%, i.e. 500 times less dissolved active. In vivo the rutin glucoside formulation increased the SPF by 27%, the rutin nanocrystal dermal formulation by 59%, i.e. two times increase at 500 times less dissolved molecules. Simplified it could be stated that the nanocrystal formulation

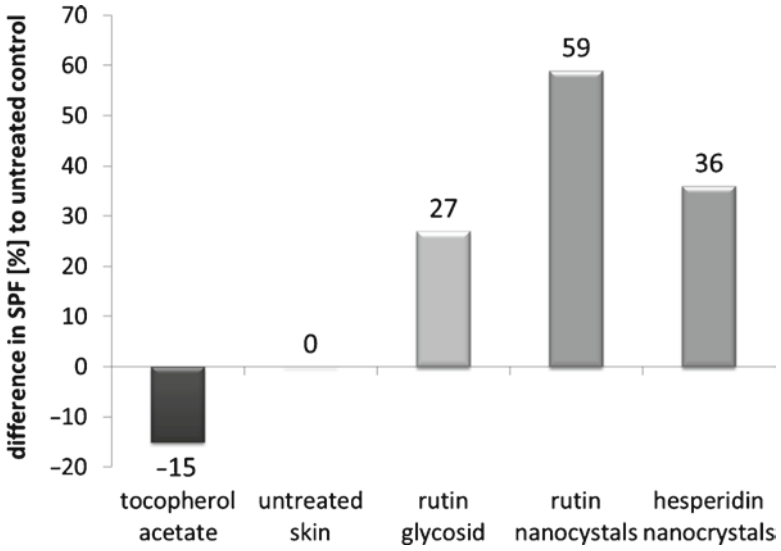

Fig. 5 Change in % of SPF of human skin after treatment with formulations containing alpha tocopherol acetate, water soluble rutin glucoside, rutin nanocrystals and hesperidin nanocrystals (After Petersen 2006, SPF of untreated skin was set = 100%)

has a 2 × 500 = 1,000 fold higher activity. A similar performance was observed for hesperidin (increase of SPF by 36%), meanwhile launched in the product platinum rare (la prairie). The alpha tocopherol acetate reduced the SPF (Fig. 5).

Explanation of the mechanism of action (Fig. 6):

1. Glucosidation made the molecule more hydrophilic, thus more water soluble. 5% could be dissolved which provided a very high concentration gradient between dermal formulation and skin. However, the hydrophilic derivative likes the hydrophilic environment in the dermal formulation, and stays there. In addition, the hydrophilic glucoside has less penetration ability than the more hydrophobic rutin.
2. The more hydrophobic rutin has a priori better penetration ability, the nanocrystals provided a supersaturated solution and consequently a concentration gradient obviously high enough for a sufficient penetration causing the antioxidant effect in the skin. Rutin penetrated into the skin was immediately replaced from rutin dissolving from the nanocrystal depot.
3. It can be assumed that the original lipophilic molecule has a higher affinity to the respective binding sites in the cell than the hydrophilic glucoside, thus being superior in antioxidant activity.
4. The observed increase in SPF is a superposition of delivery ability of the formulation and the intracellular effect of the molecule. The sum of penetration and efficiency is higher for the nanocrystal formulation, despite the 500 times less dissolved rutin in the formulation.

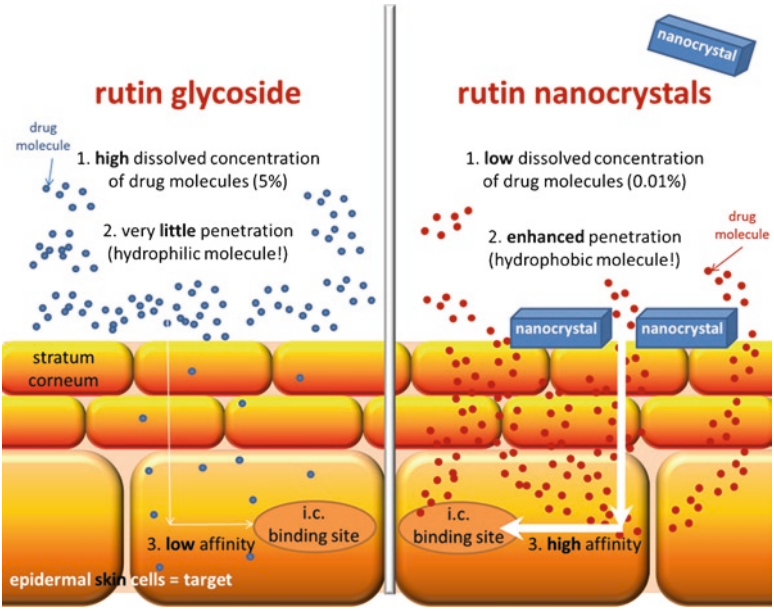

Fig. 6 Mechanism of improved dermal action of rutin nanocrystals (*right*) versus water soluble rutin glucoside (*left*, explanation cf. text)

A similar but less pronounced increase in SPF was observed for the hesperidin nanocrystals. The important conclusion is, that the nanocrystal technology opens the perspective to use new classes of molecules, e.g. the plant molecules such as flavonoids. They could not be used before because of low solubility and limited penetration of water soluble derivatives. The rutin glucoside shows some effect, but it needs to be kept in mind that 5% are needed in the formulation, and the effect being less (hesperidin), or only half of the effect of the nanocrystals (rutin). The same formulation principle can of course also be applied to pharmaceutical dermal formulations.

6.2 Pharmaceutical Market

The products on the pharmaceutical market (Table 1) exploit the same cell delivery mechanism I as the dermal products, but using the oral administration route, only one product is an injectable (Table 1). However, the delivery advantage is often combined with an increased patient convenience. Patients using more patient-friendly dosage forms show a higher compliance compared to less user friendly formulations. The principles for oral delivery are the same as for dermal. Nanocrystals provide a higher concentration gradient at the barrier, the barrier skin is replaced by

Table 1 Nanocrystal products on the pharmaceutical market: trade name, drug, producer, nanoparticle technology

Product	Drug	Company	Nanoparticle technology
Rapamune®	Sirolimus	Wyeth	Elan nanocrystals®
Emend®	Aprepitant	Merck	Elan nanocrystals®
Tricor®	Fenofibrate	Abbott	Elan nanocrystals®
Megace® ES	Megestrol acetate	PAR pharmaceutical	Elan nanocrystals®
Invega® Sustenna™	Paliperidone palmitate	Janssen	Elan nanocrystals®
Triglide™	Fenofibrate	Shionogi pharma	SkyePharma IDD®-P technology

the barrier gut wall. In addition, the nanocrystals provide a fast replacement of molecules permeated. Therefore nanocrystals are a suitable formulation for class II drugs of the biopharmaceutical classification system (BCS). Class II drugs penetrate well (similar to lipophilic rutin), but dissolve slowly, the dissolution velocity is the rate limiting step for oral bioavailability.

The first product on the market was Rapamune (sirolimus) (Müller and Junghanns 2006). The nanocrystals in a tablet have a higher bioavailability than a solution of sirolimus. In addition the solution is less patient friendly, also required special storage conditions (fridge) and complicated reconstitution. The tablet can be stored at room temperature. The second product was Emend (aprepitant, pellets in a capsule). The drug aprepitant is absorbed within an absorption window in the upper intestinal tract. The nanocrystals provide a sufficiently high dissolution velocity to exploit this absorption window for efficient oral delivery of this drug, not achievable with a classical formulation. The most successful nanocrystal product is Tricor (fenofibrate, tablet) launched by Abbott. Fenofibrate shows a 35% higher absorption in the fed state. The nanocrystal formulation removed the big difference in bioavailability between fed and non-fed state. In addition the dose could be reduced from 54/160 mg to 48/145 mg. Sales are meanwhile more than one billion USD per year, a nano block buster. Triglide is the competitive product of fenofibrate, but produced with high pressure homogenization.

Remarkable is the product Megace *ES* (*E*nhanced *S*tability), a formulation of megestrol acetate. It is a suspension with the shelf life of a pharmaceutical product. It demonstrates nicely that physically long-term stable nanosuspension can be produced – despite their state of high dispersitivity, high interfacial energy and risk of Ostwald ripening. The previous oral suspension showed a food effect on the bioavailability. The nanosuspension reduced the difference between fed and non-fed, in addition the nanosuspension is less viscous and requires only ¼ of the administration volume – three patient convenient factors increasing compliance.

Invega® Sustenna® is the first injectable nanocrystal product, approved in 2009. As once-a-month release formulation of the drug paliperidone palmitate, it is injected intramuscularly, thus avoiding the problems of i.v. injected nanosuspensions. The aqueous nanosuspension delivers the drug in a small volume, conveniently to inject.

7 Targeted Nanocrystals in Development – "Cellular Delivery Mechanism II"

In these developments the nanocrystals deliver the drug to the cells by internalization. The nanocrystals are taken up, and dissolve inside the cell (= cell delivery mechanism II). The administration route used by now is intravenous injection in form of aqueous nanosuspensions. These nanosuspensions need to be made isotonic by addition of glycerol. Addition of NaCl has to be avoided, because this reduces the zeta potential of the nanocrystals and causes subsequently aggregation. Furthermore the nanosuspensions need to be sterile, either made sterile by terminal sterilization or be produced aseptically.

At the beginning i.v. nanosuspensions were developed with the aim to replace toxicologically problematic excipients in existing i.v. formulations on the market. Examples are Taxol and Sporanox. Taxol contains paclitaxel solubilised with Cremophor EL causing sometimes anaphylactic shocks during administration (Strachan 1981; Dye and Watkins 1980). Sporanox contains itraconazol made soluble by inclusion into hydroxypropyl cyclodextrin (HP-CD). The HP-CD can cause nephrotoxicity (Szejtli 1988). The technological aim was to produce nanosuspensions of both drugs, whereas the nanocrystals are stabilized by well tolerated stabilizers, e.g. lecithins or Poloxamer 188.

Paclitaxel nanosuspensions could be successfully produced, nanocrystal size about 300 nm. Stabilizers used were well tolerable phospholipon 90 and various Poloxamers (Böhm 1999; Böhm et al. 1997). However after i.v. administration of the nanosuspension the pharmacokinetic was completely different to the solution Taxol. The drug nanocrystals were recognized as being foreign to the body and taken up the macrophages of liver and spleen. The same was observed for an injected itraconazole nanosuspension (Rabinow et al. 2007). With regard to the original development aim of a generic product, this was a failure but the data demonstrate nicely the possibility to target drugs via nanocrystals to the cells of the mononuclear phagocytic system (MPS). Of high interest are for example anti-HIV drugs to target to viruses residing in the macrophages, e.g. as shown for the drug nevirapine (Müller, Shegokar, and Keck in press).

There are two ways to imitate the pharmacokinetics of injected solutions. Firstly the nanocrystals can be made small enough that they are dissolved before "meeting" the macrophages. It was shown that i.v. injected 897 nm oridonin nanosuspensions accumulated in the liver, whereas 103 nm nanocrystals showed a pharmacokinetics similar to a solution (Gao et al. 2008). Secondly, the nanocrystal surface can be modified analogue to the stealth liposomes generating stealth nanocrystals. A stealth surface avoids the adsorption of e.g. opsonins which leads to the recognition by the macrophages. Pre-requisite of this concept is that the stealth properties on the nanocrystal surface remain during the dissolution process of the nanocrystals in the blood. The stealth properties can be checked in vitro by analysing the protein adsorption patterns in plasma and in serum (Lück et al. 1998; Lind et al. 2001; Göppert and Müller 2003). The analytical tool is two-dimensional polyacrylamide

gel electrophoresis (2-DE, 2D-PAGE) (Blunk et al. 1993) or two-dimensional differential in gel electrophoresis (2D-DIGE). At least a negative selection is possible identifying surfaces which adsorb opsonins, thus minimizing animal experiments.

Cells of the MPS are a relatively easy target, but the complexity starts when a certain MPS population should be targeted, e.g. lung macrophages. After i.v. injection recognized particles are cleared mainly by the liver macrophages, up to 90% of the injected does within 5 s, 2–5% by the spleen and only a few % by the lung macrophages (Müller 1991). Avoiding e.g. the uptake by the liver macrophages and directing the nanocrystals to macrophage subpopulations is a first challenge. One approach could be to use opsonins specific to macrophage subpopulations (Roubin and Zolla-Pazner 1979). For other cellular target sides, recognition by the MPS cells needs to be avoided completely and simultaneously a homing device attached to the surface to localize the particles at the target cells. After i.v. injection only target cells are accessible which can be reached via the blood stream.

Kreuter et al. found that Tween 80 stabilized i.v. injected polymeric nanoparticles could deliver the drug dalargin to the brain (Kreuter et al. 1997, 2003). As mechanism was identified that after injection apolipoprotein E in the blood bound to the particle surface and mediated the adherence to the endothelial cells of the blood-brain barrier (BBB) (Müller et al. 2001). For paclitaxel-loaded polymeric nanoparticles could be shown, that the drug was released in the endothelial cells and diffused from here into the brain (Gelperina et al. 2002; Kreuter 2001). However, the basic problem was that only a small part of the injected particle mass reached the brain (e.g. loss to the liver) and that the drug loading of the particles was relatively low. This resulted in low drug concentrations in the brain. It would be desirable to use a nanocarrier with a very high loading capacity, i.e. using drug nanocrystals.

This was realized with buparvaqone nanocrystal suspensions. In vitro it could be shown that the nanocrystals adsorbed apolipoprotein E. They were tested using a toxoplasmosis animal model. After i.v. injection into mice, the parasites could be completely eradicated in the brain (Schöler et al. 2001). However, cure of the animals was not achieved (Schöler 2001) under the study design applied, which can be a function of the design and/or some parasites still residing somewhere in the body. The drug loading with the nanocrystals was much higher than with polymeric nanoparticles, but after injection there was loss of drug by dissolution during their travel time to the blood-brain barrier. To minimize this drug loss, the nanocrystals should be coated with a thin polymer layer. The surface properties of the polymer layer can be designed this way that apolipoprotein E is adsorbed preferentially, may be in higher amounts than on Tween 80-stabilzed nanocrystals. For example, the nanocrystals could be coated with the polymer of the polymeric nanoparticles by Kreuter which proved efficient in targeting the endothelial cells (i.e. poly(butyl) cyanoacrylate) (Schroeder et al. 1998; Alyautdin et al. 1995).

Another concept is to use a “taxi” for the nanocrystals to deliver them into the brain. The taxi can be macrophages in the blood, which extravasate and travel e.g. to sites of inflammation, including crossing the blood-brain barrier. This concept was exploited by various research groups, e.g. Barrett Rabinow et al. (Dou et al. 2009),

and in a modified version by Bäumler et al. (Staedtke et al. 2010). They loaded Amphotericin B nanosuspension (AmB-NS) into human red blood cells (RBCs). The AmB-NS-RBCs were then taken up by phagocytosis by leukocytes, which are the main effector arms of antifungal defense. The leukocytes carry drugs also in areas of inflammation. The loading of the RBCs with nanoparticles should be preferentially performed ex vivo in purified cell populations, also the phagocytosis by the leukocytes. This avoids competitive uptake (e.g. in vivo after injection of nanosuspension by MPS organs, ex vivo by other cells present in the suspension). In case the nanocrystals should be loaded into cells which have no phagocytic capacity (e.g. RBC), the nanocrystals need to be small enough to be internalized by pinocytosis, i.e. below 100 nm. This was performed for example with Amphotericin B nanocrystals which were 65 nm in size.

Basically three targeting levels can be differentiated:

1. a specific organ
2. a certain cell population within the organ
3. and ideally a specific compartment within the cells,

whereas level three is the most challenging. To our knowledge very little work has been done to study the intracellular fate of nanocrystals.

The reason for this is surely the problem of simultaneous distribution and dissolution of the nanocrystals inside the cell. One approach to tackle this problem is the use of fluorescent nanocrystals, and studying simultaneously dissolution and resulting drug distribution inside the cell. With different nanocrystals different drug distributions should be achievable. This is definitely one field of investigation in the future.

8 Conclusions and Perspectives

Delivery to target cells and subsequent intracellular delivery by internalization of particles is under investigation for more than half a century, dating back to the 1950s. This involves identification of mechanisms to localize nanocarriers in target cells or cell compartments, and in parallel the development of suitable nanocarriers, preferentially usable in patients at the end of the day.

At the beginning targeting mechanisms investigated were very simple, e.g. in the 1960s effect of charge on i.v. injected particles (Wilkins and Myres 1966). Meanwhile the targeting mechanisms got rather complex, e.g. via antibodies or via modulation of the protein adsorption patterns in the blood to enrich the particle in a target cell. In future it will get even more complex when considering the conformation of these proteins and the role of the conformation in cellular uptake.

Parallel went the development of nanocarriers. Using the number of nanocarriers for drug delivery on the market for treatment of patients as a performance measure, the success was limited. The most important nanocarriers in the second half of the last century were i.v. nanoemulsions and liposomes. In 2000 the nanocrystals

appeared on the market. From our point of view, the nanocrystals have clear advantages compared to many of the "academic" nanocarriers presently under development. These are besides others accepted status of excipients, well tolerable (NCS class I/III), easy and cost-effective large scale production, proven make-ability of products. Tricor® is the first nano block buster, making the nanocrystals to the most successful nanotechnology by now.

The next steps will be to combine sophisticated targeting approaches with the nanocrystals as carrier and to exploit much more the opportunities in controlled intracellular delivery.

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