Chapter 11
Squalenoylation: A Novel Technology for Anticancer and Antibiotic Drugs with Enhanced Activity

Patrick Couvreur

Abstract This chapter describes the 'squalenoylation' technology, a platform for the discovery of new nanomedicines. The design of nanomedicines is generally based on the physical encapsulation, adsorption, or entrapment of a drug in a nanocarrier. This generally results in poor drug loading, and often an uncontrolled fast release of the drug (known as burst release). To overcome those limitations, the squalenoylation concept is based on the chemical (rather than physical) loading of drugs in nanomedicines. The idea is to link a biologically active compound (anticancer, antibiotic, antiviral, MRI imaging agent, etc.) to squalene, a natural and biocompatible lipid. Due to the unique, dynamically folded molecular conformation of squalene, the resulting squalene–drug bioconjugates self-assemble spontaneously in water to form nanoparticles. The resulting nanoassemblies have been shown to have enhanced pharmacological activity, and with reduced toxicity, thus paving the way to a new concept in the field of drug delivery.

Imagine a nanoparticle arriving at the surface of a sick cell to deliver drug molecules or a gene designed to reprogram the cell. This is the ultimate goal of nanometric pharmaceutical carriers, or nanocarriers. The idea was first formulated at the beginning of the twentieth century by the savant Paul Ehrlich, who referred to it as the magic bullet. However, the world had to wait until the 1970s before it was shown that artificial particles measuring a few hundred nanometres could penetrate into the heart of a cell, causing the accumulation of molecules that would not normally diffuse there: so this was the real birth of the nanocarrier.

More generally, the products of nanotechnology are not merely a miniaturisation of larger objects. Because they are nanometric, they have properties that would not be found on longer length scales. And the field of medication is no exception to this rule. Until the beginning of the 1970s, intravenous administration of pharmaceutical suspensions such as a dispersion of solid particles in a liquid was considered

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unthinkable, owing to the obvious risk of possibly fatal embolism. Today, however, nanoparticulate suspensions can be designed to carry drug molecules (nanodrugs) and thereby increase the therapeutic index of many compounds; that is, their activity is enhanced and/or their toxicity is reduced following intravenous administration. The transition from a size of a few tens of microns to a few tens or hundreds of nanometres has thus led to an important technological and medical breakthrough.

The expected advantages of nanodrugs are:

- protection of the active molecule against its own degradation,
- targeting of the active molecule to the relevant tissue, cell, or subcellular compartment (cytoplasm, lysosomes, nucleus),
- the possibility of using a contrast agent to visualise the tissue and/or the distribution of the nanocarrier while it accomplishes its therapeutic activity (theranostics).

By using 'intelligent' biomaterials, one can also arrange for these nanosystems to release the active principle they carry in response to some exogenous or endogenous stimulus, e.g., a modification of the pH or ionic strength, a change in temperature, or the application of an extracorporeal magnetic field. Hence, the slightly acidic environment of a tumour tissue can increase the number of protons H^+ in the transport material, making it soluble or changing its conformation, in such a way as to induce release of the active principle within the tumour. Drug release in the tumour can also be triggered by carrying out anti-tumoral hyperthermia, e.g., if the lipid used to carry the drug has a phase transition temperature of the order of 40–42°C. The application of an extracorporeal magnetic field is another method for achieving targeted drug delivery, provided that the drug in question is carried by a magnetic nanocarrier, e.g., containing maghemite, an iron oxide with chemical formula Fe_2O_3.

Ever more intelligent nanodrugs can thus be prepared thanks to progress in the techniques of supramolecular assembly and self-assembly, but also using new ways of functionalising the surface of nano-objects, including the methods known as click chemistry (see Chap. 5).

The applications of nanotechnology to therapeutics are thus undeniably related to the ability of nanosystems to control the release of active molecules both in time and in space. This is why these technologies apply more particularly to active molecules, whose toxicity for healthy tissues currently restricts the use of large doses. It is not therefore surprising that the therapeutic indications of nanodrugs already on the market concern the treatment of cancer (Caelyx®, Doxil®, DepoCyt®, Abraxane®) and deep mycoses (Ambisome®). Despite the considerable progress made in the field of materials chemistry and pharmaceutical formulation (galenic pharmacy), there is no escaping the fact that the nanodrugs available today remain limited to the areas just mentioned. Indeed, there are several important technological bottlenecks:

1. The low level of encapsulation of nanoparticulate systems, the weight percentage of the active principle relative to the carrier material rarely exceeding 5 %.
2. The fast release, or burst release, of the encapsulated drug which may occur before the therapeutic target is reached. This uncontrolled release is due to the fraction

of drug molecules that are simply adsorbed onto the surface of the nanocarrier and not actually encapsulated within it.
3. The difficulty in obtaining low toxicity, biodegradable synthetic materials which do not induce cell or tissue thesaurismosis.

There is thus a broad consensus that it has become urgent to propose and validate new concepts that will introduce breakthrough technologies in the field of drug delivery. This must be achieved by introducing new and more efficient biocompatible materials. The coupling of squalene with drugs currently used to treat cancer and viral pathologies is a perfect example of such an innovative technology, known as squalenoylation. It leads to more efficient nanodrugs for the treatment of these serious diseases.

11.1 Origin of the Concept of Squalenoylation

Squalene is a lipid in the terpene family. It is a naturally occurring cyclic molecule, found in large quantities in the plant kingdom, e.g., in olive oil, wheat germ, rice, and so on, and also in the animal kingdom, e.g., in shark liver oil. It is also ubiquitous in human tissues, and in particular in the skin. It can thus be considered as completely lacking in toxicity.

Squalene has the unique property of adopting a cyclic structure in lanosterol, itself a precursor of cholesterol, by spontaneously rolling up into a highly compact molecular conformation in an aqueous medium. Indeed, thanks to its molecular flexibility and compact nature, squalene can adopt various conformations which allow it to enter the hydrophobic pocket of the enzyme oxidosqualene cyclase, where the cyclisation reaction takes place. Note that, quite remarkably, this reaction occurs without the need for coenzymes and without the supply of biochemical energy in the form of adenosine triphosphate (ATP).

This unique property of squalene to adopt a highly compact molecular conformation has been exploited to obtain particulate nanosystems by coupling this lipid with anticancer or anti-infection molecules, and in particular with nucleoside analogues [1]. Squalenoylated nanodrugs obtained in this way have proved to be much more effective than their parent molecules. Indeed, these nanosystems:

1. can be administered orally or intravenously,
2. favour intracellular penetration,
3. facilitate passage through membranes,
4. protect the active molecule from degradation and/or metabolisation.

As we shall see later, the nature of the chemical bond between squalene and the drug can allow selective release of the latter at the biological target.

11.2 Coupling Squalene with Drug Molecules

Figure 11.1 is a schematic view of the chemical coupling of a squalene molecule (the carrier) with different kinds of nucleoside analogues with anticancer or antiviral activity (the drug molecules or active principles). Squalene (the chemical radical R here) is first oxidised in acid, before coupling with a given group of the nucleoside. The chemical bonding groups can be of different kinds (sugar: OH–, amine : N–, group sensitive to the surrounding pH, etc.). The derivative molecules thus obtained assemble almost miraculously into nanoparticles, either crystallised or otherwise and with sizes in the range 100–300 nm, thereby constituting the nanodrug. If the nanoparticle surface is then decorated by chains of the hydrophilic polymer polyethylene glycol (PEG), the nanocarrier can avoid otherwise rapid capture by macrophages, while at the same time being functionalised by chemical ligands able to recognise tumoral or infected cells (see Chap. 5).

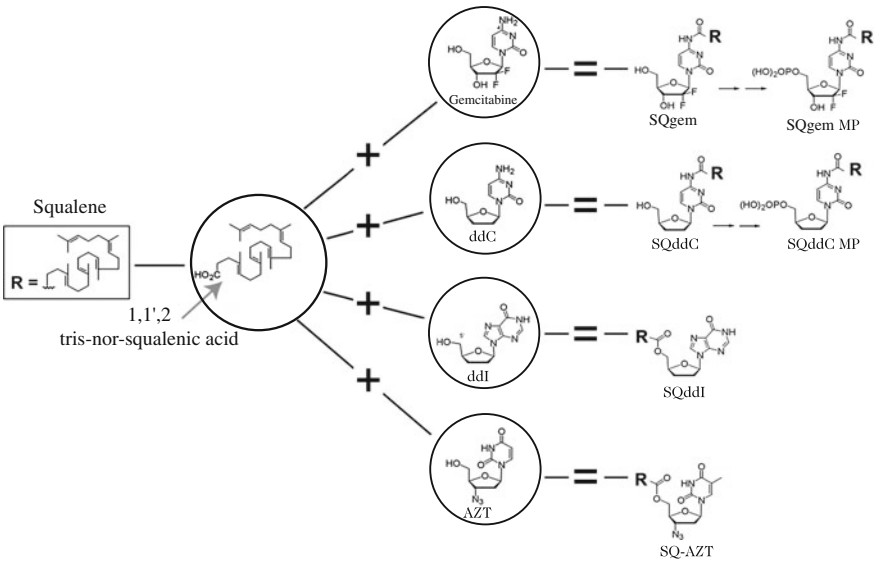

Fig. 11.1 Coupling of squalene (R) via squalenic acid to different nucleoside analogues with anticancer activity (gemcitabine) or antiviral activity [ddI (didanosine), ddC (zalcitabine), AZT (zidovudine)]. Adapted from [2], © 2012 Elsevier. Reproduced with permission

11.3 Characteristics and Morphology of the Main Squalene Derivatives

Table 11.1 lists the main drug molecules that have so far been coupled with squalene, together with the therapeutic treatments they are intended for (cancers, infectious diseases, etc.), the type of molecular coupling, and the size and organisation of the nanoparticles obtained by clustering of the squalenoylated molecules.

The examples in Table 11.1 display the extraordinarily universal and flexible nature of squalenoylation technology, which can be adapted to many molecules with therapeutic activity, be it anticancer, antiviral, or antibiotic, with a variety of physicochemical characteristics. There are hydrophilic molecules (nucleoside analogues like gemcitabine), lipophilic molecules (paclitaxel), small molecules (penicillin, etc.), and macromolecules (siRNA). In every case, the squalene conjugates self-assemble spontaneously in an aqueous medium to form nanoparticles, by virtue of molecular mechanisms that are still poorly understood. In some cases, the supramolecular organisation leads to hexagonal phases, viz., squalene–gemcitabine nanoparticles (see Fig. 11.2) or cubic phases, viz., ddC–squalene. In others, a total absence of organisation is observed, viz., penicillin–squalene or paclitaxel–squalene nanoparticles (see Fig. 11.3).

11.4 Applications to Cancer Treatment

11.4.1 Nanoparticles of Gemcitabine Coupled with Squalene

In the field of cancer treatment, squalenoylation was first applied to gemcitabine (Gemzar®) to produce SQgem, used clinically to treat solid tumours such as bronchial cancer (not small cells), either locally advanced or metastatic, pancreatic cancer, bladder cancer, and breast cancer. However, gemcitabine is quickly metabolised by the action of deoxycytidine deaminase, mainly located in the blood, the liver, and the kidney, thereby forming the uracil derivative which turns out to be totally inactive. Administered intravenously, gemcitabine thus has a non-optimal anticancer activity. In addition, despite a slightly increased lipophilicity due to the presence of two fluorine atoms, this molecule is still highly hydrophilic, and this limits its passive diffusion through the plasma membrane. This means that the intracellular penetration of gemcitabine can only be achieved by active transport using a protein, the nucleoside transporter hENT1. Inhibited expression of this transporter then often implies resistance to treatment. This happens with pancreatic cancer, for example.

When gemcitabine couples with squalenic acid, the resulting molecular derivative has the quite exceptional property that, in an aqueous medium, it can spontaneously form uniformly sized nanoparticles (130 nm) that can be administered intravenously. Preclinical trials have thus been carried out to compare the squalene–gemcitabine (SQgem) nanoparticles with free gemcitabine (gem) solutions.

Table 11.1 Main characteristics of squalenoylated nanodrugs so far reported

Drug molecule (nucleoside, antibiotic, etc.)	Expected therapeutic effect	Squalene–drug molecule coupling	Organisation resulting from coupling after precipitation
Gemcitabine (dFdC)—gem	Anticancer—cancers of the pancreas, bronchi, bladder, breast, etc.	Amine group N–	Formation of organised 100–300 nm nanoparticles. Examples: inverted Hexagonal phase for SQgem [3] (Fig. 11.2); cubic phase for SQddC [4]; several crystalline phases for doxorubicin nanoparticles, with elongated shape (nanospaghetti), inducing a remarkable vascular remanence [5]
Zalcitabine (ddC)	Antiretroviral—AIDS	Amine group N–	
Didanosine (ddI)	Antiretroviral—AIDS	Sugar group OH–	
Zidovudine (AZT)	Antiretroviral—AIDS	Sugar group OH–	
Cytarabine	Anticancer—leukemia	Amine group N–	
Acyclovir	Antiviral—herpes	Amine group N–	
Adenosine	Various biological processes	Amine group N–	
Thymidine	Various biological processes	Sugar group OH–	
Doxorubicin	Anticancer	OH– group	
Paclitaxel (mitosis inhibitor)	Anticancer—cancers of the lung, ovary, breast, head and neck, etc.	2′–OH group [6]	Formation of amorphous 100–300 nm nanoparticles
Penicillin G	Antibiotic—various bacterial strains	Ester group with bond sensitive or insensitive to pH [7]	Formation of amorphous 150 nm nanoparticles (Fig. 11.3)
siRNA (small interfering RNA)	Hydrophilic anticancer macromolecule—cancer of the thyroid	Coupling of squalene maleimide to the RNA coding strand [8]	Formation of amorphous 160 nm nanoparticles

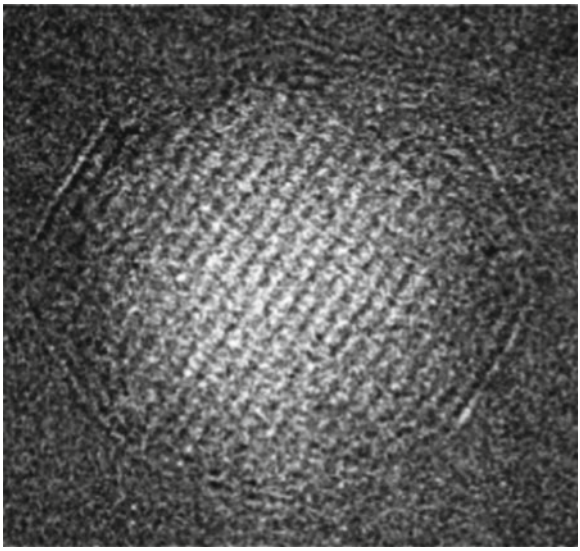

Fig. 11.2 Morphological appearance of a squalene–gemcitabine (SQgem) nanoparticle. Image obtained by transmission electron cryomicroscopy (cryoTEM). The hexagonal structure is visible. Adapted from [3], © 2008 Wiley. Reproduced with permission

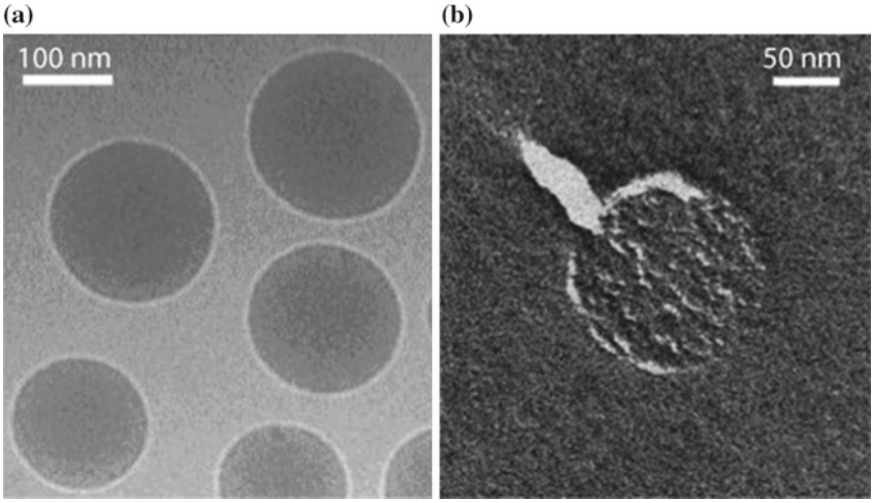

Fig. 11.3 Morphological appearance of penicillin–squalene nanoparticles (either pH-insensitive or pH-sensitive). **a** Image obtained by transmission electron cryomicroscopy (cryoTEM), not showing the internal organisation of the nanoparticles. **b** Image obtained with cryofracture for better visualisation of the internal relief of the nanoparticle. The absence of organisation is confirmed. Adapted from [7], © 2012 American Physical Society

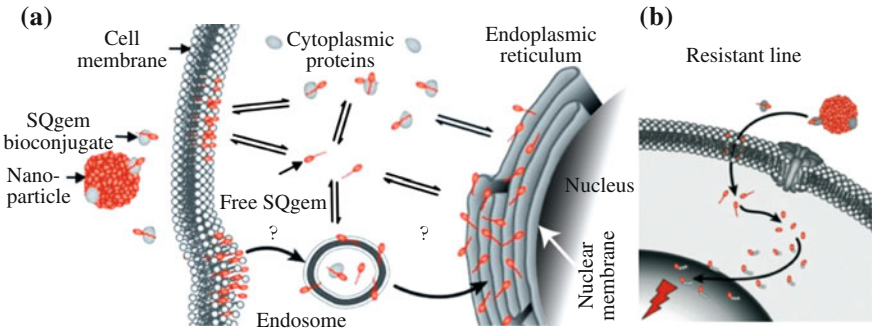

Fig. 11.4 **a** Interaction mechanism between squalene–gemcitabine (SQgem) nanoparticles and a cancer cell. After crossing the cell membrane, the SQgem bioconjugate distributes itself between the intracellular lipid membranes and the more hydrophilic cytoplasmic medium (*double arrows*). The gemcitabine is released by the action of cell enzymes and, after phosphorylation, can then reach the DNA in the cell nucleus. One question, indicated by the *question mark*, today answered in the affirmative, was whether this phosphorylation did actually occur in the cytoplasm. **b** Schematic view of the pathway followed by the gemcitabine molecules from the nanoparticle to the cell nucleus, without the need for the nucleoside transporter hENT1. The *grey-shaded* appendages of the gemcitabine molecules symbolise their phosphorylation (to gem-triphosphate). Adapted from [10], © 2010 Elsevier. Reproduced with permission

In Vitro Trials

These trials have shown that, in contrast to non-squalenoylated gemcitabine, SQgem nanoparticles are stable in the presence of blood plasma, since there is no metabolisation. They release the gemcitabine under the action of intracellular enzymes [1], according to the following mechanism (see Fig. 11.4a):

1. In the extracellular medium, the nanoparticles release SQgem in molecular form [9]. Released individually in this way, the molecules associate with extracellular proteins, favouring their diffusion toward the cell membrane where they accumulate in large quantities.
2. The SQgem molecules are then distributed between the cell membrane and the intracellular membranes (endolysosomal and endoplasmic reticulum membranes) [10].
3. The SQgem is subsequently metabolised by intracellular enzymes (cathepsins B and D), and this releases the gemcitabine in the cell cytoplasm.
4. Finally, phosphorylation of the gemcitabine by deoxycytidine kinase, then by pyridine kinases, serves to integrate the molecule into the DNA, leading to its cytotoxic effect.

When the cells are resistant to gemcitabine owing to reduced expression of the transporter hENT1, penetration of the SQgem within the cancer cell is not therefore inhibited, in contrast to what happens with free gemcitabine. This therefore provides a way round the gemcitabine resistance of the cancer cells (see Fig. 11.4b) [10].

In Vivo Trials

Pharmacokinetic trials have been carried out after intravenous administration of gemcitabine in free form or in the form of squalenoylated nanoparticles at a dose of 15 mg/kg. It was observed that the SQgem nanoparticles induced much higher plasma concentrations of gemcitabine than when the gemcitabine was administered in free form. This shows that the nanoparticulate form of gemcitabine protects the drug molecule from metabolisation and degradation. Furthermore, a biodistribution study of the labelled product showed that gemcitabine concentrations in the main organs are always higher when the anticancer agent is administered in the form of nanoparticles.

Testing the Efficacy of Squalenoylated Gemcitabine on Mouse Leukemia

The anticancer activity of SQgem nanoparticles was first tested on a mouse leukemia model (L1210) grafted intravenously on laboratory animals [11]. This is a highly aggressive metastatic model which induces fast weight loss. The animals die 20 days after the tumour cells are grafted. Treatment with free gemcitabine extends survival up to 40 days, but there are no long term survivors. No animal is cured. In contrast, treatment by SQgem nanoparticles cures 75 % of the animals, i.e., some 75 % of them survive in the long term. Moreover, tumour cell samples showed that the SQgem nanoparticles induce the arrest of cell division in phase S and a much higher level of apoptosis than after treatment by free gemcitabine. These results are explained by the better distribution of the anticancer agent in the deep organs where the metastasis takes place. Preliminary toxicological trials specified the maximal tolerated dose, and at this dose, no major toxicity was observed [11]. At higher doses, the toxicological profile (hematopoietic toxicity) is comparable with that of free gemcitabine.

Since squalene is a lipid that is particularly well absorbed orally, the efficacy of SQgem nanoparticles was also tested on a metastatic model of rats carrying the lymphocytic leukemia RNK-16 LGL. In this model, and following treatment by SQgem nanoparticles, 60 % of the animals survived in the long term, whereas no animal was cured by treatment with gemcitabine in the free form, administered orally at the same dose [1]. This result is explained by an increase in the concentration of gemcitabine in the lymphoid organs.

After intravenous administration, the SQgem nanoparticles are also much more active than gemcitabine on the mouse leukemia model P388 grafted subcutaneously (solid tumour). Following treatment by SQgem nanoparticles, the mice exhibited no visible tumour nodule, whereas a five times higher dose of free gemcitabine did not reduce the tumour mass compared with non-treated animals [12].

Testing the Efficacy of Squalenoylated Gemcitabine on Pancreatic Cancer

With a survival rate of only 3 % at five years and a median survival period of just six months, pancreatic cancer is one of the tumour pathologies with the worst prognosis. Gemcitabine is the primary treatment. This is why we tested the efficacy of SQgem nanoparticles on an orthotopic mouse model of a human pancreatic cancer (panc-1)

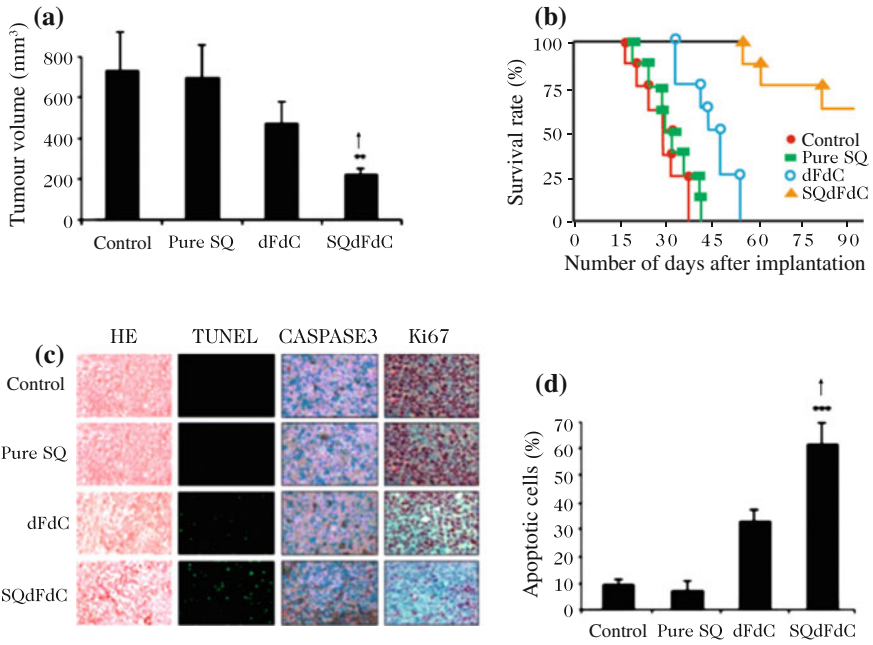

Fig. 11.5 SQgem nanoparticles (denoted here by SQdFdC) exhibit greater antitumour activity than free gemcitabine (denoted by dFdC) and pure squalene nanoparticles (pure SQ) on the orthotopic model of human pancreatic cancer panc-1. **a** Size of tumour nodules for the different treatments. **b** Survival rate of the animals for the different treatments. **c** Histology and immunohistochemistry of tumour biopsies after treatment. **d** Cells in apoptose after treatment. Adapted from [13], © 2011 Elsevier. Reproduced with permission

[13]. In this case, the tumour cells are grafted in the pancreatic head. The size of the tumour (see Fig. 11.5a) and the survival of the animals (see Fig. 11.5b) were assessed as a function of the treatment. For example, SQgem nanoparticle treatment produces a spectacular reduction in the size of the pancreatic tumour nodule, leading to a 65 % long term survival rate, whereas all the other treatments (nanoparticles of pure squalene or free gemcitabine) leave no survivors in the long term. These results are confirmed by immunohistochemistry (see Fig. 11.5c) and measurement of cancer cell apoptosis for the different treatments (see Fig. 11.5d). Indeed, the nanoparticle treatment induces an increase in the number of cells in apoptosis, viz., 60 % as compared with 30 % for the free gemcitabine treatment, while at the same time reducing the number of cells in proliferation.

Hence the bright red spots produced by staining tumour biopsies with hematoxylin and eosine (HE, see Fig. 11.5c) indicate the presence of large numbers of dead cells following treatment by SQgem nanoparticles. The Tunel labelling immunohistochemical test reveals the programmed apoptosis of tumour cells by green fluorescence (rupture of the DNA). The caspase-3 labelling test confirms the increased

number of cell deaths by a dark brown colour. On the other hand, labelling with the Ki67 antigen attests, by a bright stain (here, blue), to reduced proliferation of tumour cells due to the treatment.

It has been shown recently that it is also possible to associate two drugs in the same nanoparticle: one of them, SQgem, induces tumour cell death, while the other iso-Combretastatin, reduces tumour vascularisation. This double action has proved to be extremely effective on an experimental model of colon cancer. This is the principle of the multidrug nanoparticle [14].

11.4.2 Nanoparticles of Doxorubicin Coupled with Squalene

Doxorubicin-loaded nanocarriers have gained increasing interest as they can improve the treatment of tumours and reduce drug-mediated cardiotoxicity. However, the need to surface-functionalise conventional nanocarriers such as liposomes with PEG raises toxicological issues because PEG is not biodegradable. In this context, it has been discovered that, through a simple manufacturing procedure, the linkage of doxorubicin to squalene allows the synthesis of 130 nm non-PEGylated nanoparticles (SQ-Dox) with impressively high drug loading (i.e., 57 %), slow drug release, and a novel 'loop-tail' elongated structure never observed before [15]. Further physicochemical and morphological investigations will certainly be needed to explain these surprising results, but the elongated morphology of SQ-Dox nanoparticles is likely to be the explanation for their ability to circulate for long periods in the bloodstream after intravenous injection. The fact that 'loop-tail' SQ-Dox can align with the bloodstream makes them invisible for liver macrophage recognition.

Cell culture viability tests and apoptosis assays showed that SQ-Dox displayed about the same antiproliferative and cytotoxic effects as native doxorubicin. *In vivo* experiments showed that SQ-Dox dramatically improved anticancer treatments as compared with free doxorubicin. In particular, M109 lung tumours that did not respond to doxorubicin treatment were found to be inhibited by 90 % when treated with SQ-Dox. Similarly, SQ-Dox-treated MiaPaCa-2 pancreatic human tumours xenografted in mice were found to decrease by 95 % instead of the 29 % reduction achieved with native doxorubicin [15]. It was also shown that the maximum tolerated dose for SQ-Dox nanoassemblies was five times higher than for the free drug and that SQ-Dox did not cause any myocardial lesion such as those induced by a free doxorubicin treatment. All these results demonstrate that nanoparticles of doxorubicin coupled with squalene make tumour cells more sensitive to doxorubicin and reduce the cardiac toxicity, thus providing a remarkable improvement in the drug's therapeutic index.

11.4.3 Nanoparticles of SiRNA Coupled with Squalene (SQsiRNA)

The nucleotide sequences of fusion oncogenes are unique to the cancer cells to which they belong and represent specific targets for the development of new anticancer molecules. Hence, for papillary thyroid cancer, the most frequent endocrine tumour of the thyroid, inhibition of the fusion oncogene (RET/PTC1) should provide a way to halt the tumour process. The development of a small interfering RNA (siRNA)[1] has the advantage of precisely targeting the fusion oncogene and hence avoiding side effects due to inhibition of normal genes in non-tumour cells, as happens with non-specific treatments. Indeed, these siRNA molecules are highly promising therapeutic agents because of their specific action: they are active at low dosage and exhibit low toxicity. On the other hand, they are extremely hydrophilic macromolecules that degrade very quickly in the plasma and do not spontaneously penetrate cells.

An siRNA molecule with sequence complementary to the fusion oncogene messenger RNA was coupled to squalene maleimide (see Table 11.1). This is a novel approach because most nucleic acid nanocarriers use cationic lipids or polymers owing to the affinity of positive charges for cell membranes. However, these materials are not without a certain level of toxicity, and in particular the destruction of red blood cells (hemolysis). By coupling siRNA to a lipid like squalene, one can avoid the toxicity inherent in polycations, while taking advantage of the strong affinity of squalene for most cell membranes.

11.5 Application to Treatment of Infectious Diseases

11.5.1 Antiretroviral Nucleoside Analogues

The idea of squalenoylation has been applied to nucleoside analogues with antiviral activity, such as zalcitabine (ddC) and didanosine (ddI). However they bind to the squalene, on the sugar or the heterocycle (see Fig. 11.1), all the squalenoylated molecules self-assemble in water to form 100–300 nm nanoparticles. Their derivatives have been tested *in vitro* for their anti-HIV activity on infected lymphocytes from three different donors. The two squalenoylated derivatives proved to be two to three times more active than the corresponding parent molecules with regard to their ability to inhibit viral multiplication by 50 %.

The antiviral activity of SQddI and SQddC nanoparticles was then tested on human lymphocytes infected by certain resistant viral strains of the AIDS virus (HIV-1-144 and HIV-1-146). The antiviral activity of the squalenoylated derivatives was much greater than that of ddI or ddC on these same resistant strains. For example, SQddI nanoparticles proved to be ten times more active than ddI on the HIV-1-146 strain.

[1] Small interfering RNA (siRNA) is a short ribonucleic acid with 20 or 25 base pairs.

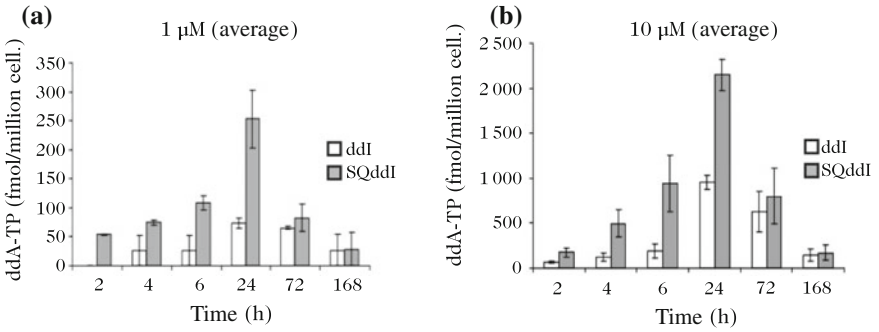

Fig. 11.6 Intralymphocyte concentrations of ddA-TP after incubation of lymphocytes with ddI (*white*) or with SQddI (*grey*) at a concentration of 1 µM (**a**) or 10 µM (**b**). Adapted from [15], © 2008 Elsevier. Reproduced with permission

It is also found that SQddI nanoparticles induce much higher intracellular concentrations of the active form of the ddI molecule, viz., ddA-TP (2′, 3′-dideoxyadenosine-5′-triphosphate), and this whatever the concentration of the product incubated with the cells. This result suggests that phosphorylation is much more efficient with SQddI nanoparticles than with ddI (see Fig. 11.6).

11.5.2 Intracellular Antibiotherapy

The treatment of intracellular infections is another promising field of application of squalenoylated nanodrugs. When they arrive in the blood compartment, the bacteria are opsonised to facilitate capture by macrophages in the body's defence system (the liver and the spleen) where they are destroyed, in particular by the lysosomal enzymes of the cells. In certain situations, such as immunodepression, opportunistic infections, and others, the lysosomes of these macrophages actually provide a kind of haven for the multiplication of intracellular bacteria. Many antibiotics are largely inactive against these germs located within the cell, either because they are degraded in the intracellular medium, or because they do not diffuse easily inside the cells, or again because they are rapidly washed out of the cells and never reach the infected intracellular compartments (endosomes or lysosomes) in sufficient concentrations. In other words, the infected macrophages end up as reservoirs of bacteria that are resistant to most conventional antibiotic treatments because these only eradicate extracellular germs. There is thus a real need to develop antibiotic nanocarriers targeting the intracellular environment.

This has been done by coupling penicillin to squalene by both pH-sensitive and pH-insensitive links. When the link is pH sensitive, the release of the antibiotic is triggered by the acidic pH of the infected lysosomes [7]. These pH-sensitive or pH-insensitive penicillin–squalene nanoparticles penetrate the macrophages (J777) by

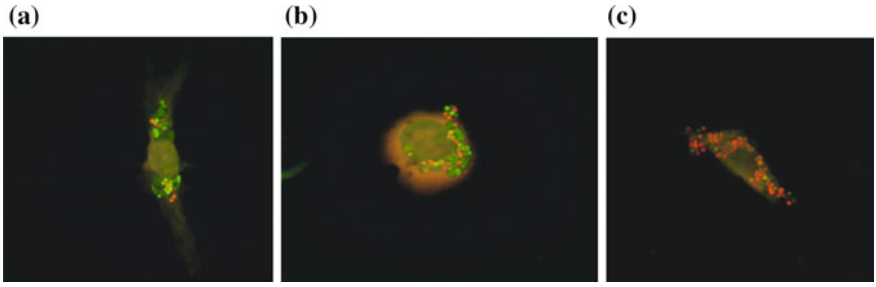

Fig. 11.7 Fluorescence confocal microscopy showing living (*green*) and dead (*red*) intracellular bacteria. **a** Non-treated macrophages. **b** Macrophages treated with free penicillin. **c** Macrophages treated with penicillin–squalene nanoparticles. Adapted from [7], © 2012 American Physical Society

endocytosis and end up in the cell lysosomes. The antibacterial activity then proves to be much more effective in the case of pH-sensitive penicillin nanoparticles [7]. Indeed, on a model of macrophages (J774) infected by *Staphylococcus aureus*, these nanoparticles can very quickly kill the intracellular bacteria (see Fig. 11.7), proving that nanotechnology can provide effective tools for treating resistant intracellular infections.

11.6 Application to the Treatment of Neurological Disorders

Drug delivery remains the main challenge in central nervous system (CNS) drug development, due to the rapid metabolisation and/or rapid blood clearance of most CNS drugs, and sometimes poor diffusion through the blood–brain barrier (BBB) and the blood–spinal cord barrier (BSCB). A typical example of the problems that can arise for drug delivery in neurological pathologies is adenosine. Indeed, this molecule represents a class of potential therapeutic agents with significant beneficial activity in severe neurological disorders such as strokes, spinal cord injury, or multiple sclerosis. However, adenosine exhibits serious limitations due to its short plasma half-life (a few seconds) following rapid metabolisation, the advent of moderate side-effects, and its inability to cross the BBB and BSCB, and for these reasons, it has never been used for the treatment of neurological diseases.

Notwithstanding, a very simple and easy way to use the currently unusable adenosine as a neuroprotective drug with intravenous injection is the conjugation of adenosine with squalene (SQAd) and the subsequent formation of nanoassemblies allowing prolonged circulation of this nucleoside. This approach has been shown to provide neuroprotection in a mouse stroke model and a rat spinal cord injury model [21]. The animals receiving systemic administration of SQAd nanoassemblies showed a significant improvement of neurological deficit score in the case of cerebral ischemia, and an early motor recovery of the hind limbs in the case of spinal cord injury

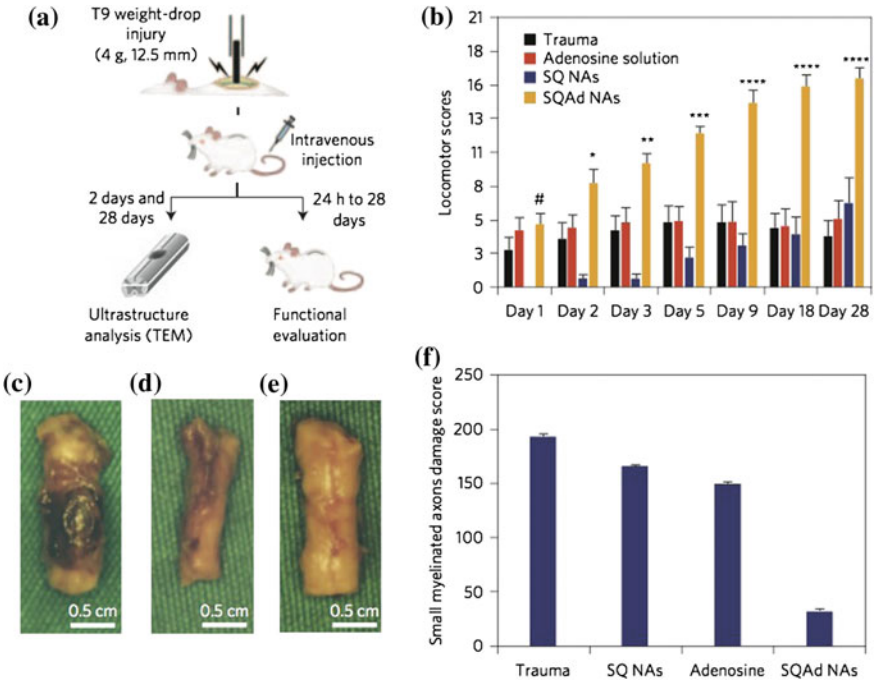

Fig. 11.8 Pharmacological efficiency of the SQAd nanoassemblies in a model of spinal cord injury in rats. The pharmacological efficiency of the squalene–adenosine nanoassemblies (SQAd NA) was assessed in a T9 contusion spinal cord injury model. Within 5 min, the animals were intravenously injected with either dextrose 5 %, SQAd NAs, or free adenosine (**a**). After 24, 48, and 72 h, and up to 28 days post-trauma, the animals were functionally graded using the Basso, Beattie, and Bresnahan grading (**b**). After 72 h, the SQAd NA injected animals showed a complete recovery of their hind limbs, in accordance with the absence of any visible traumatic area on the cord (**e**) compared to trauma group (**c**) and the adenosine treated group (**d**). Quantification of the damage to the small myelinated axons showed that the SQAd NAs dramatically reduced the damage score (**f**) compared to all other groups. Reprinted from [21] by permission from Macmillan Publishers Ltd

(see Fig. 11.8). Moreover, in FRET experiments (see Sect. 2.7.2), it has been demonstrated for the first time that nanoparticles can provide neurological protection despite the fact they are unable to translocate the blood–brain barrier. The reason is that these nanoparticles can favour a peripheral vascular mechanism, leading in turn to secondary parenchymal neuroprotection [21].

11.7 Magnet Guidance, Imaging, and Theranostics

Iron oxide particles (magnetite) can be included in the core of the nanocarrier in such a way that it will respond to an extracorporeal magnetic field. This idea has been used to guide gemcitabine in a transplanted mouse tumour model. In practice,

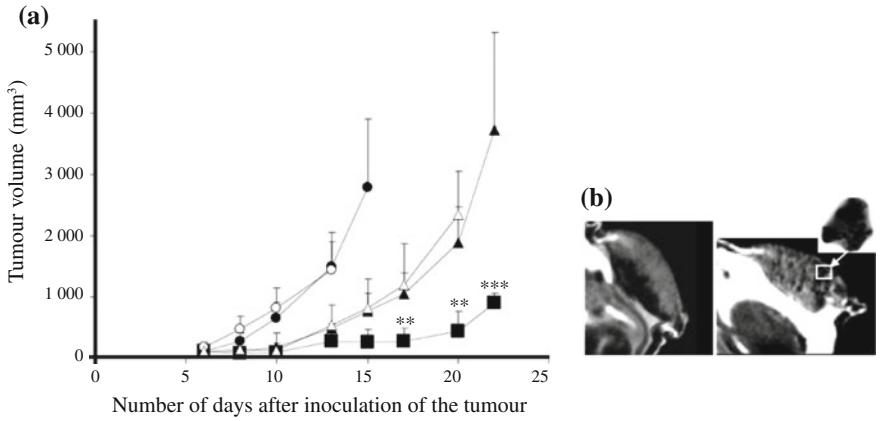

Fig. 11.9 **a** Evolution of the tumour volume: (●) no treatment, (○) gemcitabine, (▲) SQgem nanoparticles, (△) SQgem/USPIO nanoparticles without application of an extracorporeal magnetic field, (■) SQgem/USPIO nanoparticles with application of an extracorporeal magnetic field of 1.1 T. **b** Visualisation of the tumour nodule by MRI after injecting SQgem/USPIO nanoparticles without (*left*) and with (*right*) application of an extracorporeal magnetic field. The reduction in the T2 signal due to the presence of USPIO shows up as *dark spots* within the tumour (*insert*). Adapted from [17], © 2011 American Physical Society

ultrasmall magnetic nanoparticles (ultrasmall superparamagnetic iron oxide USPIO) measuring only a few nanometres, are trapped within a nanomatrix made from SQgem molecules and having dimensions of the order of a hundred or so nanometres [16]. These nanosystems are then injected intravenously while a magnetic is placed close to the tumour. The inhibition of tumour growth is spectacular and can be monitored by magnetic resonance imaging (MRI) thanks to the presence of the iron oxide particles which, within the lipid nanomatrix, induce a reduction in the MRI T2 signal [17] (see Fig. 11.9).

The core of the nanocarrier thus plays both a therapeutic and a diagnostic role (nanotheranostics). This still highly experimental idea has been extended to other molecules with cytotoxic activity, such as paclitaxel, doxorubicin, and cisplatin, and also to other contrast agents, in particular squalene coupled with gadolinium. The technique can thus be adapted to many active principles and contrast agents. The association of a drug with an imaging tool within the same nanocarrier provides an ideal route to personalised medicine. Indeed, being able to monitor both the nanocarrier distribution by MRI and at the same time also its therapeutic efficacy by visualising the tumour size means that it will be possible to make a clinical decision about stopping or pursuing the treatment on a case-by-case basis.

11.8 From Squalenoylation to Terpenoylation

As mentioned in Sect. 11.2, squalene belongs to the terpene (or isoprene) family, which is an extremely diverse group of compounds, both with regard to chemistry and from the structural and functional point of view [18]. Their lipophilic nature and their permanent presence in living organisms mean that they are likely to play a role in the elaboration of the most primitive biological membranes [19]. Surprisingly, although most terpenes, both natural and synthetic, are easy to handle and biocompatible polymers, and despite the fact that their physicochemical characteristics allow them to adapt to a wide range of active principles, these compounds have never been used to transport and deliver drugs, with the exception of squalene, whose self-assembly into nanoparticles was discovered by researchers at the *institut Galien* of the Paris-Sud university.

The aim now is therefore to extend the idea of squalenoylation to terpenoylation by chemically coupling a biologically active molecule to different kinds of terpenes in order to identify which bioconjugates will or will not be able to self-assemble to form nanoparticles in an aqueous medium. For example, gemcitabine has been coupled to several terpenes containing one to six isoprene units. Figure 11.10 shows the chemical structure of one isoprene unit. To get an idea, squalene contains 6 isoprene units and generally speaking there are many possible isoprene chains for any given number of units.

In the first *in vitro* experiments, it was observed that the anticancer activity of the nanoparticles was closely linked to the size of the polyisoprene chain. Nanoparticles containing three isoprene units (farnesyl) proved to be the most effective on different lines of tumour cells. Trials carried out *in vivo* on an experimental xenograft model of human pancreatic cancer confirmed the anticancer activity of certain nanoparticles of gemcitabine–farnesyl derivatives (leading to 76 % tumour inhibition) and gemcitabine–squalene nanoparticles (leading to 41 % tumour inhibition), whereas free gemcitabine proved to have no antitumour effect on this model. The level of anticancer activity was in fact correlated to the gemcitabine release kinetics. These observations show that the cancer cells can be made more sensitive to the gemcitabine treatment by modulating the length of the polyisoprene chain, and this without conspicuous toxicity.

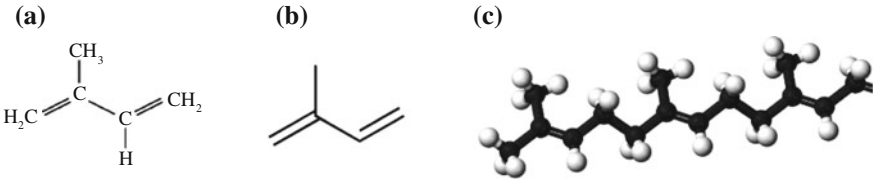

Fig. 11.10 **a** Chemical structure of an isoprene unit. **b** Simplified representation of one unit. **c** Three-dimensional representation of farnesyl containing three isoprene units

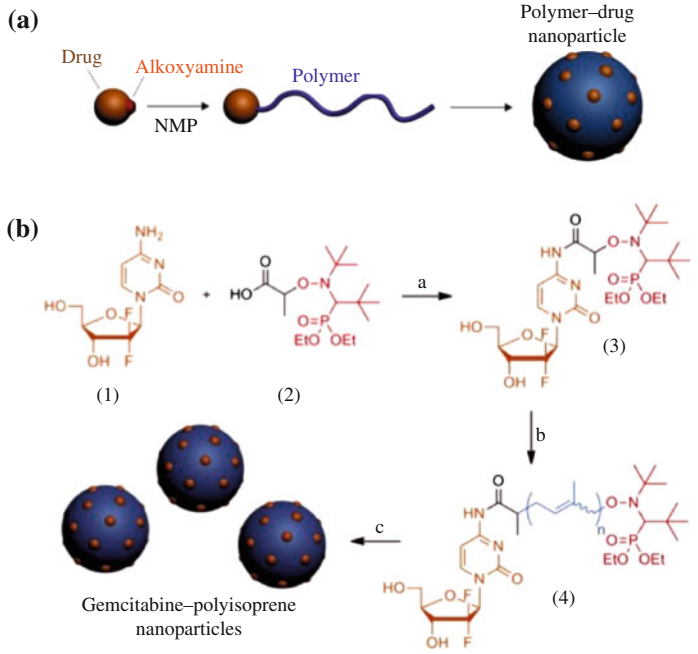

Fig. 11.11 Construction of gemcitabine–polyisoprene nanoparticles. **a** General principle of controlled radical polymerisation from a drug serving as a trigger for the polymerisation reaction. This produces a conjugate in which the end of each polyisoprene chain is coupled to a drug molecule. The macromolecule thus obtained forms stable nanoparticles in water. **b** Application to gemcitabine carrying a macro-alkoxyamine group. The reaction (*1*) + (*2*) ⟶ (*3*) followed by polymerisation (*4*) leads to a gemcitabine–polyisoprene conjugate which forms stable nanoparticles in water. Adapted from [20], © 2013 Wiley. Reproduced with permission

Recently, with a view to increasing the number of isoprene units beyond six, researchers at the *institut Galien* have developed a novel way of building nanoparticles using longer polyterpene sequences [20]. This approach is based on chemical modification of the anticancer agent using an alkoxyamine function to initiate controlled isoprene polymerisation (see Fig. 11.11b). This so called nitroxide mediated polymerization (NMP) is a radical polymerisation reaction providing good control over the macromolecular characteristics of the polymer. For example, the length of the polyisoprene chain attached to the gemcitabine can be easily adjusted to produce the most effective nanoparticles. The excellent anticancer activity of the nanoparticles obtained from a gemcitabine–polyterpene derivative of mean molecular weight 2 510 dalton (corresponding to more than 35 isoprene units) has been demonstrated [20]. This coupling strategy based on controlled growth of a hydrophobic oligomer from an anticancer drug carrying a macro-alkoxyamine group leads to an anticancer agent at each end of the synthesised polyterpene chain (see Fig. 11.11a). This discovery, which could be applied to other active principles than gemcitabine, looks likely to extend the available pharmacological applications.

11.9 Conclusion

The idea of squalenoylation, and its extension to terpenoylation, provide an example of a highly original nanotechnology platform for the design of new anticancer, neuroprotective, and anti-infection nanodrugs. Constituting as it does a completely innovative approach, this highly flexible technique may well meet major societal needs by opening the way to new prospects for the treatment of cancers, neurological disorders, or infections that resist conventional practice. Researchers at the *institut Galien* where this work was initiated have already developed a first type of nanodrug for the treatment of resistant forms of hepatocarcinoma, and it is currently undergoing phase III clinical trials, the last phase prior to industrialisation, by the company BioAlliance, now ONXEO. This innovation has just been awarded the European Inventor Award 2013, thus illustrating the dynamical relationship between fundamental research and the pharmaceutical industry in the field of nanodrug development. As in many similar cases, the transition from research to pre-industrialisation can only be made through company start-ups, while continuing to maintain the work of the research group, with the support of both public and private funding, and taking care to patent all inventions before publishing research results in the scientific journals.

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