CHAPTER FIVE

Invention of Novel Drug Delivery Systems for Improvement and Enhancement of Chemotherapy

Nanoparticles of Biodegradable polymers for new-concept chemotherapy

Clinical trials are the most important step for any medical product to be approved for clinical use. Pharmacology of experimental animals could be very different from that of humans, and interpatient and intrapatient differences also exist. *Few reports of clinical trials can be found from the literature for nanoparticles of biodegradable polymers for chemotherapy, although some preclinical guidelines have been provided.* Chemotherapy plays an important role especially when surgery and radiotherapy fail to cure the patients, who have only a 10% chance of cure by other therapies.[65]

While the effort to find medical solution should be continued, new hope will most likely come from emerging technology, especially nanobiotechnology, which can be defined as chemotherapeutic engineering.[66] The situation for the emerging chemotherapeutic engineering looks

[65] Feng S. S. (2004). Nanoparticles of Biodegradable polymers for new-concept chemotherapy. Expert Rev. Medical Devices 1 (1), pp.115-125.
[66] Feng SS, Chien S. (2003), Chemotherapeutic engineering: application and further development of chemical engineering principles for chemotherapy of cancer and other diseases. Invited review. Chem. Engr. Sci. 58, 4087-4114.

similar to that for tissue engineering 30 years ago. Chemotherapeutic engineering will be well defined and begin to play a necessary and important role in the fight against cancer and other fatal diseases in the next 5 years.

It has been shown by the marvelous progress in the past decades in molecular biology, materials science and nanoparticle technology, that nanoparticles of biodegradable polymers have great potential to provide an ideal solution for most of the major problems encountered in chemotherapy and with further development, to promote a new concept of chemotherapy, which may include sustained chemotherapy controlled and targeted chemotherapy, personalized chemotherapy, chemotherapy across various physiological drug barriers such as the GI barrier for oral chemotherapy and the BBB for treatment of brain tumors and other CNS diseases, and eventually, chemotherapy at home. Chemotherapy will become safer, more efficient and eventually under full control. The quality of life of the patents can then be greatly improved. While tissue engineering is thought to be likely to change the traditional concept of surgery, chemotherapeutic engineering should be of potential to substantially change the current practice of internal medicine.

Development of effective carriers for both existing and newly developed anticancer drugs may be as important as the discovery of new anticancer drugs.

There has been intense research over the past decade into the development of nanoparticles of biodegradable polymers as effective drug delivery systems for chemotherapy. Progress in nanoparticle technology, material

science and engineering, and cellular and molecular physiology and pathology has contributed to the advancement in nanoparticle technology for chemotherapy. The polymers used are biocompatible and biodegradable, either synthesized or natural, which are subject to FDA approval. The drug can either be dispersed in the polymeric matrix, or conjugated/attached to the polymer molecules. Following administration, the drug can be released from the nanoparticles. The drug release mechanism can be diffusion, polymer matrix swelling, polymer erosion and degradation. For most FDA-approved biodegradable polymers, which have bulk erosion properties, drug diffusion and polymer matrix swelling play a major rule since the degradation of these polymers is relatively slow, often occurring over a year or so. However, more and better polymers of surface erosion properties or hydrophilic components are being developed, where polymer erosion/degradation can play a primary role. No other adjuvant is required. The drug encapsulated in the nanoparticles will gradually be released from the polymer matrix, which will eventually be degraded into harmless molecules such as hydrogen, nitrogen and water. Nanoparticles of biodegradable polymers can be made adequately small to allow intracapillary or transcapillary passage and can be appropriately coated to escape elimination by the reticuloendothelial system, as well as to promote adhesion to and uptake by cancer cells. Nanoparticles for cancer chemotherapy have been extensively investigated in the past decade, including, but not limited to, paclitaxel, doxorubicin and 5-FU.

In addition to drug formulation, nanoparticles of biodegradable polymers can be employed to solve other problems in chemotherapy such as pharmacokinetics, drug toxicity and drug resistance.

Polymeric nanoparticles can be prepared either by dispersion of the polymers or by polymerization of monomers; both approaches involve the use of chemical engineering techniques. Various FDA-approved biodegradable and biocompatible polymers such as polylactic acid (PLA), polylactic-co-glycolic acid (PLGA) and polyepsilon-caprolactone (PCL) are available for this purpose.

NEW DRUG DISCOVERY - BIRTH OF A DRUG

From the time it leaves the discovery laboratory until it is cleared by the U.S. Food and Drug Administration, a new drug typically follows a series of well-defined steps. Here is an overview of the process that all pharmaceutical companies must follow to file a new compound in the United States.[67]

Development of new treatments in medicine is made possible by the design, conduct and reporting of prospective clinical trials. There are number of early stage and internal

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[67] Roche Pharmaceuticals in U. S. (2002). Innovative Research and Development - Birth of a Drug.
Elan Corporation, (2001- 2004). Benefits of Drug Delivery. Dublin, Ireland.

development projects for each of the technology platforms which are shown below:

- **Feasibility** – In vitro (laboratory) feasibility study to determine whether, under laboratory conditions, the formulation of the product candidate can be achieved. Laboratory and animal studies are conducted to evaluate the safety and efficacy of the new compound in lower animals, to determine its toxicity and clinical effects.
- **Investigational New Drug Application (IND) –** to receive permission in the U.S. to test the drug on humans, companies, show the results of previous series of animal testing. Further pharmacological and pharmacokinetic experiments then attempt to elucidate the method of action of the drug and suggest appropriate doses for administration to humans.
- **Phase I** - Also called *pre-clinical*, where batches are manufactured for in vivo studies (in humans) in healthy volunteers. This phase involves testing on 20 to 80 normal, healthy volunteers to determine a safe dosage and how the drug is absorbed and metabolized in the human body. Some preliminary dose proportionality data may be obtained. At this phase, the drug development group may set about developing an appropriate dosage form for administration to humans.
- **Phase II** – Also called pre-pivotal trials. Additional in vivo testing my be performed involving in small patient population. In phase II clinical trials, patients, affected by the disease for which the therapeutic indication is being sought, are recruited to gain information on the dose proportionality of the drug, and preliminary

efficacy data. It is at this phase that a dosing schedule is usually determined.

- **Phase III** – Also called pivotal trials. Phase III clinical trials are larger programs in which the product is administered to an expanded patient population typically at dispersed sites. All of the improved outcome or new products under development require a phase III trial. To assess the drug's effectiveness, studies are conducted with 1,000 to 3,000 patients, with the disease that the drug has been designed to treat. Physicians monitor patients closely to confirm the drug's efficacy and identify adverse reactions. The purpose is to determine the safety and effectiveness of the drug when compared with a placebo or an established product on the market.
- **Commercial Manufacturing -** In parallel with Phase II and Phase III trials, development work is usually undertaken to scale up the production of the prototype dosage forms developed in the laboratories.
- **New drug Application (NDA) – Filed.** Data from all phases are analyzed and findings (if positive) are compiled and filed with the FDA. Company (which initially develops original production) file for regulatory approval in jurisdiction in which it is intended that the product will be marketed. For example, in USA, this will require filing with the FDA.
- **FDA Advisory Review -** An independent panel of experts appointed by the FDA reviews the NDA, considers presentations by company representatives and FDA reviewers, then makes a recommendation to the

FDA. The FDA may or may not follow that recommendation.

- **Labeling Discussions -**Companies work with the FDA on the specific wording for the product label, which provides the essential information needed by a physician to prescribe a drug properly.
- **Registration -** Information collected throughout the discovery and development process is forwarded to a government regulatory agency (FDA, in the United States) for a complete review. The agency determines, sometimes with the help of a committee of experts, whether the company submitting the file has made a case for use of the drug in patients for specific diseases. In particular, the FDA will look for statistical and clinical evidence that the drug is effective, often compared to drugs currently used in the market, and most importantly, that it is safe for use in the indication which has been studied. If the company has shown this convincingly, the drug will then be approved.
- **Approval**– Approved by the relevant regulatory authority.
- **Phase IV – Marketed.** Product is available in the market. Once a drug has been cleared for marketing, the new medicine is made available to physicians to prescribe. Once marketed, further clinical studies may be undertaken to compare the market potential or cost-effectiveness of the new drug with established market leaders, or in other therapeutic areas for which marketing claims are not sought. These studies are usually termed "Phase IV trials," and they are used to

position the product in the marketplace and facilitate acceptance of the new drug among the medical community. Phase IV studies are often undertaken to answer questions posed by regulatory authorities and thought leaders. The company must continue to submit periodic reports to FDA, including any cases of adverse reactions.

Drug Delivery Systems

Devices capable of bypassing biological barriers to deliver therapeutic agents with accurate timing and at locally high concentrations directly to cancer cells will play a critical role in the development of novel therapeutics.

Drug delivery and targeting systems under development aim to minimize drug degradation and loss, prevent harmful side effects and increase the availability of the drug at the disease site. Drug carriers include micro and nanoparticles, micro and nanocapsules, lipoproteins, liposomes, and micelles, which can be engineered to slowly degrade, react to stimuli and be site-specific. Targeting mechanisms can also be either passive or active. An example of passive targeting is the preferential accumulation of chemotherapeutic agents in solid tumors as a result of the differences in the vascularization of the tumor tissue compared with healthy tissue. Active targeting involves the chemical ‘decorating’ of the surface of drug carriers with molecules enabling them to be selectively attached to diseased cells.

The controlled release of drugs is also important for therapeutic success. Controlled release can be sustained or pulsatile. Sustained (or continuous) release of a drug involves polymers that release the drug at a controlled rate, by diffusion out of the polymer or by degradation of the polymer over time. Pulsatile release is often preferred, as it closely mimics the way by which the body naturally produces hormones such as insulin. It is achieved by using drug-carrying polymers that respond to specific stimuli (e.g. exposure to light, changes in pH or temperature).

Other nano-based approaches to drug delivery are focused on crossing a particular physical barrier, such as the blood-brain barrier; or on finding alternative and acceptable routes for the delivery of a new generation of protein-based drugs other than via the gastro-intestinal tract, where degradation can occur. Nanoscience and nanotechnology are thus the basis of innovative delivery techniques that offer great potential benefits to patients and new markets to pharmaceutical and drug delivery companies.

For over 20 years, researchers in Europe have used nanoscale technology as the basis of vast improvements in drug delivery and targeting, and Europe is now well placed to build on this body of knowledge [68].

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[68] European Technology Platform on NanoMedicine. Nanotechnology for Health. Vision Paper and Basis for Strategic Research Agenda for NanoMedicine. European Commission. September 2005.

Benefits of Drug Delivery

There is enormous potential for nanotechnology to be applied to gene and drug delivery. The vehicle might be a functionalized nanoparticle capable of targeting specific diseased cells, which contains both therapeutic agents that are released into the cell and an on-board sensor that regulates the release. Different stages of this approach have already been demonstrated, but the combined targeting and controlled release have yet to be accomplished. In this event the way will be opened up for initial trials, and the eventual approval of such techniques will be fully regulated as for any new pharmaceutical.

A related approach already in use is that of polymer-based drug therapies: they include polymeric drugs, polymer–drug conjugates, polymer–protein conjugates, polymeric micelles to which the drug is covalently bound, and multi-component complexes being developed as non-viral vectors for gene therapy. Many of these materials are now undergoing clinical trials for a variety of disease states[69].

Polymeric nanoparticle have advantages over liposomes and micelles and the polymer-based drug delivery systemss (DDS) have relatively shorter history . It is thus clear that more and better results for polymer synthesis nanoparticle formulation and characterization and *in vitro*

[69] The Royal Society & The Royal Academy of Engineering (2004), Nanoscience and nanotechnologies: opportunities and uncertainties, Bulletin: Nanoscience and nanotechnologies.

and *in vivo* experiments, are needed for nanoparticles of *biodegradable polymers to be approved for clinical trials as a DDS* for chemotherapy of cancer and other diseases such as cardiovascular restenosis. It is most likely that clinical trials of nanoparticles for chemotherapy could be approved and would be under intensive investigation if and only if close collaborations between oncologists and biomedical engineers have been established. It is probable that nanoparticles of biodegradable polymers will become commercially available as a medical device in the next 5-10 years[70].

Perspectives of drug delivery systems

The concept of polymeric DDS was first introduced in the early 1960s by Folkman and Long[71]. In the 1970s, the long-term controlled release of contraceptive steroids, narcotic antagonists, local anesthetics, antimalarial and anticancer agents were mainly investigated to attain potentiation of the pharmacological activities and elimination of the inconvenience of repeated injections. Thereafter, in the late 1980s, biodegradable polymers were investigated intensively for those peptides and proteins to both achieve satisfactory efficiency and increase patient

[70] Feng S. S. (2004). Nanoparticles of Biodegradable polymers for new-concept chemotherapy. Expert Rev. Medical Devices 1 (1), pp.115-125.

[71] Juliano, R.L ., (1980).Drag delivery systems: characteristics and biomedical applications, Oxford University Press.

compliance. [72] Primarilyin vitro studies DDS investigated for various applications. Brem et al.[73] reported the delivery of nitrosourea carmustine (BCNU) from biodegradable polyanhydride disks. The DDS can be either biodegradable by using degradable polymers such as poly(lactide-co-glycolide) (PLGA),[74, 75, 76, 77, 78] fibrin,[79] and

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[72] Xudong Cao (1997). Delivery of Neuroactive Molecules from Biodegradable Microsperes. M.A. Sc. Degree thesis. Department of Chemical Engineering and Applied Chemistry. University of Toronto.

[73] Brem, H., Tamargo, R. J., Olivi, A., Pinn, M., Weingart, J. D., Wharam, M., Epstein, J.I. (1994). Biodegradable polymers for controlled delivery of chemotherapy with and without radiation therapy in the rnonkey brain, J. Neurosurg., 80,283-290.

[74] Okada, H., Doken, Y., Ogawa, Y., Toguchi, H. (1994). Sustained suppression of the pituitary-gonadal axis by Leuprorelin three month depot microspheres in rats and dogs, Pharmaceutical Res., 11,1199-1103.

[75] Yamakawa, I., Tsushima, T., Machida, R., Watanabe, S. (1992). In vitro and in vivo release of poly (DL-lactic acid) microspheres containing neurotensin analogue prepared by novel oil-in-water solvent evaporation method, J. Pham. Sci., 81, 808-811.

[76] Gupta, P.K., Johnson, H., Allexon, C. (1993). In vitro and in vivo evaluation of clarithromycin/poly(1actic acid) microspheres for intramuscular drug delivery, J. Controled Release, 26, 229-238.

[77] Arshady, R. (1991). Preparation of biodegradable microspheres and microcapsules. Polyactides and related polyesters, J. Controlled Release, 17, 1-22.

[78] Arshady, R. (1990). Biodegradable microcapsular drug delivery systems: manufacturing methodology, release control and targeting prospects, J. Bioactive Compatible Polymers, 5, 315-342.

bovine serum albumin; or biostable by using non-biodegradable polymers such as poly(ethylene oxide) (PEO) and ethylene-vinyl acetate copolymer (EVAc). Injectable and biodegradable microspheres appear to be a particularly ideal delivery systems because they are small and they degrade, obviating the necessity to remove the device after the drug supply is exhausted.

Microspheres and microcapsules

Microspheres are fine spherical particles that contain drugs that can be divided into two categories: (i) homogeneous or monolithic microspheres in which the drug is dissolved or dispersed throughout the polymer matrix and (ii) reservoir-type microspheres in which the drug is surrounded by the polymer matrix in a mononuclear state. Particles belonging to (i) and (ii) are referred to as microspheres and microcapsules, respectively. There is, however, no clear-cut between these two, because the morphological structures are sometimes mixed. For example, in some systems, most of the drug core is surrounded by the polymer but some drug molecules are dispersed separately or adsorbed on the surface of the polymer.

Types of Copolymers - Poly (lactide/glycolide). Linear polyesters of lactide (PLA) and glycolide (PGA) have been used for more than three decades for a variety of

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[79] Ho, HO., Hsiao, CC., Sokoloski, T. D., Chen, CY., Sheu, MT. (1995). Fibrin-based drug delivery systems, the evaluation of the release of macromolecules from microbeads, J. Controlled Release, 34, 65-70.

medical applications[80]. They were among the first synthetic degradable polymers to find application as surgical suture materials, controlled drug release devices, as well as orthopedic and reconstructive implants[81]. Low molecular weight PLA and PGA are prepared by the direct condensation of lactic acid and glycolic acid, respectively, whereas poly(lactide-co-gtycolide) (PLGA) is obtained by the condensation of both lactic and glycolic acids with or without catalysts. High molecular weigh polymers are produced by the ring opening method with a catalyst such as dialkyl zinc. Having an asymmetric carbon atom, lactic acid has two optical isomers. Therefore, its polymer consists of L-, D- and D, L- lactic acid in which the L- or D-polymers have a crystalline form and D, L- polymers are amorphous and more rapidly degraded.

Degradation of PLA, PGA, PLGA copolymers has been studied extensively both *in vitro* and *in vivo*. Degradation can be followed by changes in mass, molecular weight, morphology, mechanical strength. It has been shown that the degradation of PLA, PGA, PLGA is due to the hydrolysis of the ester bonds along the backbone of the

[80] Domb, A. J. (1994). Implantable biodegradable polymers for site-specific drug delivery in "polymeric site-specific pharmacotherapy" (A.J. Domb, Ed.), John Wiley & Sons Ltd.

[81] Menei, P., Benoit, J. P., Boisdron-Celle, M., Fournier, D., Mercier, P., Guy, G. (1994). Drug targeting into the central nervous system by stereotactic implantation of biodegradable microspheres, Neurosurgery, 34, 1058-1064.

polymer chains, and the degradation products are carboxylic acids and alcohols.

Once a polymer device is immersed into water, the first event that happens is water uptake. This results in the bulk water penetration throughout the polymer's amorphous domains whereas crystalline domains remain intact because they are less accessible to water. Due to the hydrolysis of the ester bonds along the polymer chain, the molecular weight of the degrading polymer decreases. As the degradation time increases, some of the degraded polymers are cleaved so small that they eventually become water soluble and leach out of the polymer matrix, resulting in the mass loss of the degrading device. Random scission of the ester bonds also results in a mechanical strength decay. The water impermeable domains, however, degrade much more slowly, giving rise to a multimodal molecular weight distribution as observed by gel permeation chromatography (GPC) for a degrading semi-crystalline polymer.

In general, factors that affect water uptake and hydrolysis reaction of the ester bond will affect the degradation of polymers, *i.e.* chemical structures, degree of crystailinity, molecular weight, degradation conditions *(e.g.* pH and temperature) and the size of degrading device.

Furthermore, two phenomena are of critical importance in considering the degradation of PLGA. First, degradation causes an increase in the number of carboxylic acid chain ends that are known to autocatalyse the ester bond hydrolysis. Second, only oligomers which are water soluble in the surrounding aqueous medium can escape from the matrix. It therefore can be predicted that during

degradation, soluble oligomers that are close to the surface can leach out once they are produced, whereas those which are located well inside the matrix may remain entrapped and contribute to the autocatalytic effect. The autocatalytic effect, in turn, results from the production of the carboxylic acid groups, resulting in faster degradation. This ultimately results in a steep mass loss observed in the PLGA degradation profile. A subsequent sudden release of degradation products *in vivo,* can render a sudden local environment acidic and induce an inflammatory reaction or even tissue necrosis. Recum et al. successfully employed a blend of different molecular weight PLAs to address this problem.

Biocompatibiliiy

PLA, PGA, PLGA have good biocompatibility and now are being used clinically in human therapy, such as sutures and bone fracture devices. Visscher et al. studied the tissue reaction to PLGA (50:50) microspheres by injecting the microspheres intramuscularly. There was no inflammatory reaction to the microspheres after 56 days of injection when microspheres were completely absorbed, although a few foreign body cells and the encapsulation of microspheres by immature fibrous connective tissue were observed in the first several days after injection. It was concluded that PLGA (50:50) is a biocompatible material. Another experiment was conducted by Menei et al. in an attempt to extend the application of degradable PLGA polymers into neurosurgery. The brain tissue's reaction to the injected PLGA (50:50) microspheres was investigated. PLGA (50:50) microspheres were stereotactically injected

into the rat brain. An astrocytic proliferation, which is typically found following damage to the CNS was observed, and some foreign-body giant cells were also found. However the inflammatory and macrophagous reaction decreased dramatically after 2 month and almost disappeared after 2 months when the microspheres were totally degraded. They concluded that PLGA (50:50) is a biocompatible material for implantation in the brain.

Chemical syntheses of biodegradable polymers

One of the strategies to solve problems of global environmental pollution with fossil resources wasted products is thorough recycling of polymeric materials. In such cases it would be indispensable to use biodegradable polymers, one way of their recycled is by microorganisms without consumption of thermal energy. [82]

Biodegradable polymers are defined as polymers that are degraded and catabolized, eventually to carbon dioxide and water, by microorganisms (bacteria, fungi, etc.) under natural environment. These polymers, when they are degraded, should not generate any substances that are harmful to the natural environment. Biodegradable polymers are classified into three major categories: (1) polyesters produced by microorganisms, (2) polysaccharides and other biopolymers, (3) synthetic polymers, particularly aliphatic polyesters. Synthetic biodegradable polymers have a great

[82] Okada, M. (2002). Chemical syntheses of biodegradable polymers. Progress in Polymer Science N 27, p. 87-133.

advantage, since recent advances in polymer science and technology have made it possible to design and synthesize at will a great variety of polymers with desirable properties. Furthermore, they are adaptable for mass production. As to synthetic biodegradable polymers, aliphatic polyesters are the representatives. Nowadays, aliphatic polyesters such as poly(epsilon caprolactone), poly(L-lactide), poly(butylene succinate) are commercially produced, and their output continues to increase.

Polyesters from lactides

Polylactides are widely used for medical purposes such as sutures, fracture fixation, oral implant, and drug delivery microspheres. Polylactides including polyglycolide are hydrolyzed at a relatively high rate even at room temperature and neutral pH without any help of enzymes if moisture is present, are hydrolyzed in our body to the respective monomers and oligomers that are soluble in aqueous media, and hence they are often called bioabsorbeble polymers rather than biodegradable polymers. Poly(lactic acid) (PLA) and its copolymer with glycolic acid (PLG) have increasing importance also as materials for the preparation of microspheres. The knowledge of their degradation process is important to prepare microparticulate delivery systems with suitable drug release rates. Giunchedi et al.[83] studied the degradation of a poly(D,L-lactide-co-

[83] Giunchedi, P., Conti, B., Scalia, S., & Conte, U. (1998). In vitro degradation study of polyester microspheres by a new HPLC method for monomer release determination. *Journal of controlled release*, *56*(1-3), 53-62.

glycolide) (50:50) (PLGA). They showed that the preparation methods play an important role in determining the degradation behavior of microspheres: the unloaded spray-dried particles were characterized by a higher monomer release rate than the microspheres obtained by solvent evaporation.

Market View of Commercially Available Biodegradable Polymers

In the Roadmap Report ("NanoroadSME") by authorship of *René de Groot & Dr. Jonathan Loeffler, 2006,* the focus is on the actual State of the Art and the future use of nanomaterials in the Health & Medical Systems sector.[**84**] It will give to small and medium sized enterprises (SMEs) a good tool to have a concise description of the development in this sector and to make choices for their strategy. This roadmap report has the main purpose to help SMEs which are in the process of looking for new materials with improved properties to be integrated in their new products and to give them a first list of relevant nanomaterials they should consider depending on the industrial applications foreseen, the time to market and the R&D capacity of the company.

The results of the roadmap are based on a database with information about more than 100 nanomaterials, which

[84] Groot, de R., Loeffler, J., Sutter. S., (2006). Roadmap Report Concerning the Use of Nanomaterials in the *Medical and Health Sector.* The Sixth Framework Programme of European Community.

was developed in the frame of the EC-funded project NanoRoadSME. The database and the linked roadmapping tool were structured taking into account the results of a European Survey on more than 300 European SMEs, the results of several R&D surveys and industrial SWOT analysis as well as workshops and experts' interviews. The report is structured in domains of applications in which nanomaterials have the potential to play an important role in the future in the Medical & Health sector.

Presentation of relevant nanomaterials are presented by drawing 4 tables, which represent the nanomaterial roadmap for the specific domain of application and which give the following information: the level of development of the nanomaterials and a prognosis of its evolution in the next 15 years; the timeframe of possible industrial applications in this domain at short (0-2 years), middle (3-5 years) and long term (6-10 years); the nanomaterial costs and its possible evolution at short, middle and long term (when available); the market size of the nanomaterials (when available).

For each relevant nanomaterial, detailed information like a short material description, improved properties, advantages/disadvantages of the nanomaterial, barriers for the development as well as specific applications for each category are given. Finally a list about companies and organizations (if available) active in the specific areas is presented.

In order to have a quick overview of the development stage of the different nanomaterials and its evolution, five different levels of development were defined:

1. Scientific result / technology invention ;
2. Laboratory prototype;
3. Industrial demonstrator;
4. Industrialization;
5. Market entry (ME) – the final stage in the development process. The material is now ready available for the end consumer, probably still not everywhere and at a rather higher price.

These stages of development have different order of importance for SMEs depending to their position in the supply chain of nanomaterials. Three main categories can be defined: developer of nanomaterials; producer of nanomaterials; user of nanomaterials.

Marketing description of poly-lactic-co-glycolic acid (PLGA) nanofibers

Polymer nanofibers, nanocapsules and nanofilms with good chemical and mechanical properties, used for tissue engineering and drug delivery. Description of material properties which have been improved: The morphology of the electrospun nanofibers can change with the spinning conditions, forming droplets when the concentration of the polymer solution is low.

Advantages: biodegradable; biocompatible; flexibility for nanofibers and nanofilms, nanostructured PLGA enhances cell proliferation and adhesion.

Disadvantages: high collagen type I proliferation but not so high with collagen type II or III; fabrication of PLGA complexes is needed in these cases.

Barriers for the development:

1. Technology - PLGA based materials should be further developed for specific tissues to enhance its properties.

2. Market - Many other new materials with similar applications.

3. Regulatory - Investigation in different mammals must be done before using any products in humans, although many other PLGA experiments have already been done.

4. Environmental impacts - Effects of small nanoparticles entering the human body and accumulating in the cells of the respiratory or other organ systems are yet unknown.

Table 1. Level of development of the PLGA and a prognosis of its evolution in the next 15 years in the drug delivery and tissue engineering sectors.

	Short term 2006-2012	**Middle term 2013**	**Long term 2014-2020**
poly-lactic-co-glycolic acid (PLGA) nanofibers		**Market Entry**	

Table 2. Possible industrial applications of the PLGA in the drug delivery and tissue engineering sectors in dependence of a short (0-2 years), mid (3-5 years) and long term (5-10 years) view.

	Unspecified	**0 -2 years**	**3 -5 years**	**6 -10 years**
poly-lactic-co-glycolic		**drug delivery nanocapsules**	**nanofibers hoses**	

acid (PLGA) nanofibers		**and nanospheres, nanofibers scaffold**			

Table 3. Expected market size and material costs until poly-lactic-co-glycolic acid (PLGA) nanofibers 2015.

	Short term			**Middle term**			**Long term**		
	2006	**2007**	**2008**	**2009**	**2010**	**2011**	**2012**	**2013**	**2014**
Market size poly-lactic-co-glycolic acid (PLGA) nanofibers	**~250 tons/year**			**~500 tons/year**			**~2500 tons/year**		
Material costs poly-lactic-co-glycolic acid (PLGA) nanofibers	**~37000 Euro per Kg**			**~33000 Euro per Kg**			**~29000 Euro per Kg**		

Nano- and Microparticles as Controlled Drug Delivery Systems

Nano/micro- particles occupy unique position for their attractive properties in drug delivery technology. Some of the current trends in this area will be discussed.[85]

The solvent evaporation process is commonly used to encapsulate drugs into poly(lactide-co-glycolide)

[85] Ravi Kumar, M. N. V. (2000). Nano and Microparticles as Controlled Drug Delivery Devices, J Pharm. Pharmaceut. Sci, 3(2):234-258.

microparticles (PLGA) [86]. It is well known that the candidate drugs must be soluble in the organic phase. In the case, where the active ingredient is not oil soluble, other alternative can be considered. The W/O/W-multiple emulsion method is particularly suitable for the encapsulation of highly hydrophilic drugs. For drugs which are slightly water soluble, like IdUrd (2mg/ml), other approaches must be investigated to achieve significant encapsulation: dissolution of the drug in the organic phase through the use of a cosolvent or dispersion of drug crystals in the dispersed phase. In the latter case, it is often admitted that the suspension of crystals in the organic phase can lead to an initial drug release, which is difficult control [87, 88]. To reduce IdUrd particle size, two-grinding processes were used, spray-drying and planetary ball milling [89, 90, 91].

[86] Benoit, J. P., Marchais, H., Rolland, H. and Van de Velde, V. (1996). Biodegradable microspheres: advances in production technology. In: Benoit, S. (Ed.), Microencapsulation methods and industrial applications, Marcel Dekker, New York, pp. 35-72.

[87] Bodmeir, R., Chen, H., Davidson, R. G. W. and Hardee, G. E. (1997). Microencapsulation of antimicrobial ceftiofur drugs. Pharm. Dev. Technol., 2: 323-334.

[88] Shenderova, A., Burke, T. G. and Schwendeman, S. P. (1997). Stabilization of 10-hydroxycampto- thecin in poly(lactide-co-glycolide) microsphere delivery vehicles. Pharm. Res., 14: 1406-1414.

[89] Gubskaya, A. V., Lihnyak, Y. V. and Blagoy, Y. P. (1995). Effect of cryogrinding on physico-chemical properties of drugs.I. Theophylline: evaluation of particle sizes and the degree of crystallinity, relation to dissolution parameters. Drug Dev. Ind. Pharm., 21: 1953-1964.

The optimal conditions of grinding were studied through experimental design and the impact on in vitro drug release from PLGA microspheres was then examined. More recently, Geze et al [92], studied IdUrd loaded poly(D,L-lactide-co-glycolide) (PLGA) microspheres with a reduced initial burst in the in vitro release profile, by modifying the drug grinding conditions. IdUrd particle size reduction has been performed using spray drying or ball milling. Spray drying significantly reduced drug particle size with a change of the initial crystalline form to an amorphous one and lead to a high initial burst. Conversely, ball milling did not affect the initial Id Urd crystallinity. Therefore, the grinding process was optimized to emphasize the initial burst reduction. The first step was to set qualitative parameters such as ball number, and cooling with liquid nitrogen to obtain a mean size reduction and a narrow distribution. In the second step, three parameters including milling speed, drug amount and time were studied by a response surface analysis. The interrelationship between drug amount and milling speed was the most significant factor. To reduce particle size, moderate speed associated with a sufficient

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[90] Annapragada, A. and Adjei, A., (1996). Numerical simulation of milling processes as an aid to process design. Int. J. Pharm., 136: 1-11.

[91] Villiers, V. M. and Tiedt, L. R. (1996). An analysis of fine grinding and aggregation of poorly soluble drug powders in a vibrating mill. Pharmazie, 51: 564-567.

[92] Geze, A., Verier-Julienne, M. C., et al. (1999). Development of 5-iodo-2'-deoxyuridine milling process to reduce initial burst release from PLGA microparticles. Int. J. Pharm., 178: 257-268.

amount of drug (400-500 mg) was used. IdUrd release from microparticles prepared by the O/W emulsion/extraction solvent evaporation process with the lowest crystalline particle size (15.3 μm) was studied to overcome burst effect. In the first phase of drug release, the burst was 8.7% for 15.3 mm compared to 19% for 19.5 μm milled drug particles (Geze et al).

In the other procedure, Rojas et al.[93] optimized the encapsulation of β-lactoglobulin (BLG) within PLGA microcaparticles prepared by the multiple emulsion solvent evaporation method. The role of the pH of the external phase and the introduction of the surfactant Tween 20, in the modulation of the entrapment and release of BLG from microparticles, was studied. Better encapsulation of BLG was noticed on decreasing the pH of external phase to a value close to the PI of BLG, however, a larger burst release effect. In contrast, the addition of Tween 20 increased the encapsulation efficiency of BLG and considerably reduces in the burst release effect. In addition, Tween 20 reduced the number of aqueous channels between the internal aqueous droplets as well as those communicating with the external medium. Inventors claimed that these results constitute a step ahead in the improvement of an existing technology in controlling protein encapsulation and delivery from microspheres prepared by the multiple solvent evaporation method (Rojas et al).

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[93] Rojas, J., Pinto-Alphandary, H., et al, Optimization of the encapsulation and release of β-lactoglobulin entrapped poly(D,L-lactide-co-glycolide) microspheres. Int. J. Pharm., 183: 67-71, 1999.

Blanco-Prieto et al.[94] studied the in vitro release kinetics of peptides from PLGA microspheres, optimizing the test conditions for a given formulation, which is customary to determine in vitro/in vivo correlation. The somatostatin analogue vapreotide pamoate, an octapeptide, was microencapsulated into PLGA 50:50 by spray drying. The solubility of this peptide and its in vitro release kinetics from the microspheres were studied in various test media. The solubility of vapreotide pamoate was approximately 20-40 μg/ml in 67 mM phosphate buffer saline (PBS) at pH 7.4, but increased to 500-1000 μg/ml at a pH of 3.5. At low pH, the solubility increased with the buffer concentration (1-66 mM). Very importantly, proteins (aqueous bovine serum albumin (BSA) solution or human serum) appeared to solubilize the peptide pamoate, resulting in solubilities ranging from 900 to 6100 μg/ml. The release rate was also greatly affected by the medium composition. The other results are, m PBS of pH 7.4 only 33+1% of the peptide was released within 4 days, whereas, 53+2 and 61+0.95 were released in 1% BSA solution and serum respectively. The type of medium was found critical for the estimation of the in vivo release. From their investigations, it was concluded that the in vivo release kinetics of vapreotide pamoate form PLGA microspheres following administration to rats were qualitatively in good agreement with those obtained in vitro

[94] Blanco-Prieto, M. J., Bewsseghir, K., et al. (1999). Importance of the test medium for the release kinetics of a somatostatin analogue from poly(D,L-lactide-co-glycolide) microspheres. Int. J. Pharm., 184: 243.

using serum as release medium and sterilization by γ-irradiation had only a minor effect on the in vivo pharmacokinetics (Blanco-Prieto et al).

In a recent study from England, methods used to microencapsulate human serum albumin (HSA) in a biodegradable polymer were compared for their effects on the physicochemical characteristics of HSA-loaded microparticles and on the release and integrity of encapsulated HSA. Postemulsification spray-drying enables efficient preparation of high-quality PLGA microparticles for protein drug delivery. [95]

"The polymer used was poly(D,L-lactide-co-glycolide) (75:25) (PLGA) (Boehringer Ingelheim, Resomer RG 752, MW 20,900)," noted M.E. Lane et al. at the University of London. "Microparticles were formulated by (i) w/o/w emulsification and freeze-drying (EFD) or (ii) w/o/w emulsification and spray-drying (ESD)."

In the Department of Chemical and Biomolecular Engineering, National University of Singapore, was conducted other study: Fabrication of controlled release devices using supercritical antisolvent method.[96] Supercritical antisolvent with enhanced mass transfer (SASEM) method is used to process biodegradable and biocompatible polymer PLGA (poly DL lactic co glycolic

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[95] Lane, M.E. et al. (2006). Influence of postemulsification drying processes on the microencapsulation of Human Serum Albumin. International Journal of Pharmaceutics; 307(1):16-22.

[96] Lee, L. Y., Smith, K. A., Wang, C. H., (2005). Fabrication of controlled release devices using supercritical antisolvent method.

acid) in an attempt to fabricate micro or nano sized particles for encapsulation of drugs for purposes of controlled release. In this process, an ultrasonic vibrating surface provides the liquid atomization in the supercritical fluid medium. The ultrasonic vibration also creates turbulence in the supercritical phase and enhances the mixing and mass transfer between the organic solvent and supercritical antisolvent. The setup has been designed for visualization of the liquid atomization and antisolvent process in the high pressure vessel. PLGA particles obtained from this process are further analyzed using SEM (Scanning Electron Microscopy). Experiments were also carried out to study the droplet size distribution from ultrasonic liquid atomization using a Phase Doppler Particle Analyzer (PDPA).

Drug delivery to the CNS

Due to poor transport across the BBB, when administered by an implantable drug delivery system (DDS) can be delivered directly to the CNS. Moreover, the DDS maintained the drug dosage within a desired therapeutic range for a pre-determined time via a controlled release mechanism. The treatment of infiltrating brain tumors, particularly oligodendrogliomas, requires radiotherapy, which provides a median survival of 3.5-11 years.[97] Since

[97] Daumas-Duport, C., Tucker, M. L., Kolles, J. H., Cervera, P., Beuvon, F. and Varlet, P., Oligodendrogliomas. Part II: a new grading system based on morphological and imaging criteria. J. Neuro-Oncol., 34: 61-78, 1997.

5-iodo-21-deoxyuridine (IdUrd) is a powerful radio sensitizer[98], the intracarnial implantation of IdUrd loaded microparticles within the tumor might increase the lethal effects of γ-radiations of malignant cells having incorporated IdUrd. The particles can be administered by stereotactic injection, a precise surgical injection technique[99]. This approach requires microspheres of 40-50 μm in size releasing in vivo their content over 6 weeks, the standard period during which a radiotherapy course must be applied.

In vitro study at C6 Glioma Cells of anticancer drug doxorubicin in PLGA-based microparticles

Doxorubicin (DOX), also known as adriamycin, is an anthracycline drug commonly used in cancer chemotherapy. Unfortunately, its therapeutic potential has been restricted by its dose limited cardiotoxicity and the resistance developed by the tumor cells to the molecule after some time of treatment. Like many other drugs used to treat cancer, DOX is a potent vesicant that may cause extravasations and necrosis at the injection site or any site

[98] Djordjevic, B. and Snuybalski, K. (1960). Genetics of human cell lines. Incorporation of 5-bromo and 5-iodo-deoxyuridine into the deoxyribonucleic acid on human cells and its effect on radiation sensitivity. J. Exp. Med., 112: 509-531.

[99] Menei, P., Benoit, J. P., Biosdron-celle, M., Fournier, D., Mercies, P., Guy, G. (1994). Drug targeting into the central nervous system by stereotactic implantation of biodegradable microspheres. Neurosurgery, 34: 1058-1064.

that the skin is exposed to. One way to overcome these problems is to encapsulate the drug in poly (D,L-lactide-*co*-glycolide) (PLGA) microparticles. This paper investigates the release characteristics of DOX from polymeric carriers fabricated using the spray-drying technique. Finally, a cytotoxicity test was performed using Glioma C6 cancer cells to investigate the cytotoxicity of DOX delivered from PLGA microparticles.[100]

PLGAs have different properties according to the variation of lactide and glycolide ratio. Accordingly, an increase in the lactide/glycolide ratio makes the polymer more hydrophobic thus reducing degradation rate. This can be verified by the release curve where the initial burst and release rate decreased by approximately 10% when the lactide/glycolide ratio was increased. Despite the slight improvement in the initial burst and release rate, 70% of the drug was released within the first day.

Particle size also affects the initial burst of the drug. When particle size increases, the initial burst reduces. This is because a smaller particle size has a larger volume-to-surface area. Hence, during particles formation, the low solubility of DOX in EA resulted in the easy exclusion of the drug from the polymer matrix, which resulted in drug accumulation on the particle surface.

Hence, for a smaller particle, more drug accumulates on a smaller surface area hence resulting in a greater initial

[100] Lin, R. Ng, LS., Wang, CH., (2005). In vitro study of anticancer drug doxorubicin in PLGA-based microparticles. Biomaterials 26, 4476-4485.

burst. The biconcave morphology was observed to accelerate the drug release. The morphology change can certainly affect the surface area/volume ratio. Presumably, an increase of this ratio results in the observed acceleration of drug release rate.

Conclusions

In the study performed in Singapore National University, nano/micro-particulate drug delivery devices were developed for DOX using spray drying. These devices were fabricated using various polymers, namely, PLGA, pluronic and PLLA in the form of polymer blends and composite nano/microparticles. The polymers were used in different combination with the aim of obtaining a device with improved drug release characteristics and enhanced cytotoxicity against cancer cells.

It has been found that the cytotoxicity of DOX to Glioma C6 cancer cells is enhanced when DOX is delivered from PLGA polymeric carrier. The use of a biodegradable polymer matrix, whith encapsulated DOX, reduces the toxic effects against normal cells whilst increasing its therapeutic activity.

Results of Phase II Clinical Trials with use of Implantable 5-FU-Bio-Microspheres

The group of French scientist (Faisant, N., Benoit, J. P., Menei, P.) received patent in May, 2006. Title is: “Use of Biodegradable Microspheres That Release an Anticancer Agent for Treating Gliobastoma”. United States Patent No

7,041,241. United States Patent and Trade Mark Office. This is successful achievement after conducting of series of experiments during more then decade. [101,102,103]

The present invention relates to the use of biodegradable microspheres that release a radiosensitizing anticancer agent for producing a medicament to be used simultaneously with, separately from or spread over time with a radiotherapy, for treating glioblastoma. The use of said biodegradable microspheres according to the invention results in a patient survival time of at least 90 weeks, a therapeutically effective concentration being maintained in the parenchymatous area throughout this time. The microspheres used preferably contain 5-fluorouracil of the tumor, by intratissular injection. The radiotherapy targeting the tumorous mass is dosed at 60 Gy over approximately 6 weeks. The invention also relates to a method for producing

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[101] Menei, P., Venier, M. C., Gamelin, E., Saint-André, J. P., Hayek, G., Jadaud, E., Fournier, D., Mercier, P., Guy, G., Benoit, J. P. (2000). Local and sustained delivery of 5-fluorouracil from biodegradable microspheres for the radiosensitization of glioblastoma. A pilot study. Cancer V. 86, 2 , P. 325 – 330.

[102] Menei, P., Jadaud, E., Faisant, N., Boisdron-Celle, M., Michalak, S., Fournier, D., Delhaye, M., Benoit, J. P., (2003). Stereotaxic implantation of 5-fluorouracil-releasing microspheres in malignant glioma. A Phase I study. Cancer, V. 100, 2 , P. 405 – 410.

[103] Menei, P., Capelle, L., Guyotat, J., Fuentes, S., Assaker, R., Bataille, B., Francois, P., Dorwling-Carter, D. Paquis, P., Bauchet, L., Parker, F., Sabatier, J., Faisant, N., Benoit, J. P. (2005). Local and Sustained Delivery of 5-Fluorouracil from Biodegradable Microspheres for the Radiosensitization of Malignant Glioma: A Randomized Phase II Trial. Neurosurgery. 56(2):242-248.

the biodegradable microspheres by emulsion-extraction, and to a suspension containing the biodegradable microspheres obtained using this method [104].

The polymer is preferably poly(D,L-lactic acid-co-glycolic acid), or PLGA, which is a biodegradable polymer permitted in the formulation of sustained-release galenic preparations. The poly(D,L-lactic acid-co-glycolic acid) is preferably 50:50 PLGA (i.e. containing an equal amount of lactic acid and of glycolic acid).

This study was a phase I/II open pilot clinical trial comparing the effect of perioperative implantation of 5-fluorouracil-releasing microspheres followed by radiotherapy in patients with gross total resection of high-grade glioma.

Results

The preliminary results regarding survival could not be interpreted statistically due to the small number of patients. They were, however, very encouraging. At the final assessment, in the first group treated (70 mg), the three patients died at 61, 114 and 125 weeks. It should be noted that the patient who died at 114 weeks died of pulmonary metastases of the glioblastoma. In the second group treated (132 mg), three patients died at 31, 59 and 82 weeks and two were still in remission at 159 and 172 weeks, at the date of drafting of these preliminary results.

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[104] Boisdron-Celle, M., Menei, P. H., & Benoit, J. P. (1995). Preparation and characterization of 5-fluorouracil-loaded microparticles as biodegradable anticancer drug carriers. *Journal of pharmacy and pharmacology*, *47*(2), 108-114.

The survival median for the patients is 98 weeks (it is 50.6 weeks in the literature for patients satisfying the same criteria (Devaux et al, 1993)[105]. Five out of eight patients, i.e. 62%, were alive at 18 months, whereas, in the literature, for patients satisfying the inclusion criteria of this study, the survival at 18 months is 20% (Devaux et al, 1993).

Conclusions

1. Nanotechnology provides wide opportunities to modify basic chemotherapeutic drugs, some of them are under investigation at different stages of pre-clinical and clinical trials.
2. The thorough survey of the appropriate literature revealed, that recently has been finished 2nd phase clinical trials in neurooncology with the use of 5-FU embedded in PLGA microsperes and entering 3rd phase of clinical trials. Scientist from France received patent in May 2006 by USPTMO (United States Patent and Trade Mark Office).

Guidelines for neuro-oncology - Standards for investigational studies - reporting of clinical trials

Most comprehensive and upgrade guidelines for conduction of 1 and 2 phase clinical trials in neuro-oncology are presented by Chang S. et al (2005)[106]. Authors present

[105] Devaux, B. C., O'Fallon, J. R., Kelly, P. J. (1993). Resection, biopsy and survival in malignant glial neoplasms, J. Neurosurg, 78: 767 -775.

[106] Chang, S. M., Reynolds, S. L., Butowski, N., Lamborn, K.R., Buckner, J. C., Kaplan, R. S. and Bigner D.D., (2005): GNOSIS: Guidelines for neuro-oncology: Standards for investigational studies -

guidelines to standardize the reporting of phase 1 and phase 2 neuro-oncology trials. The guidelines are also intended to assist with accurate interpretation of results from these trials, to facilitate the peer-review process, and to expedite the publication of important and accurate manuscripts. Our guidelines are summarized in a checklist format that can be used as a framework from which to construct a phase 1 or 2 clinical trial. Development of new treatments in oncology is made possible by the design, conduct, and reporting of prospective clinical trials. The phase 3 randomized, controlled clinical trial is considered to be the "gold standard" of clinical research, providing the most reliable method for comparing standard with experimental therapies. However, most clinical reports in the neurooncology literature are of phase 1 and phase 2 trials, which are required for testing the safety and efficacy of a proposed drug before the initiation of a phase 3 study. Incomplete, unclear, or inaccurate design, interpretation, and reporting of the results from these vital early phase trials can hamper timely drug development and lead to erroneous conclusions as to efficacy (Mariani and Marubini, 2000[107]). Recently, there has been a trend in the scientific community toward the creation of guidelines for increasing the transparency of clinical study results. Some examples of these guidelines include the STARD, or Standards for Reporting of

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reporting of phase 1 and phase 2 clinical trials. J. Neuro-Oncology, N 7, pp. 425–434.

[107] Mariani, L., and Marubini, E. (2000) Content and quality of currently published phase II cancer trials. J. Clin. Oncol. 18, 429–436.

Diagnostic Accuracy, statement for reporting studies of diagnostic accuracy (Bossuyt et al. 2003[108]), the TREND, or Transparent Reporting of Evaluations with Nonrandomized Designs, statement for reporting nonrandomized public health interventions (Des Jarlais et al., 2004[109]), and most familiar, the CONSORT, or Consolidated Standards of Reporting Trials, statement for the reporting of randomized, controlled clinical trials (Altman et al., 2001[110]). The CONSORT statement has particular relevance to neuro-oncology. However, as it deals exclusively with phase 3 trials, it does not address many important issues that arise in the reporting of phase 1 and phase 2 neuro-oncology trials.

CONSORT, or Consolidated Standards of Reporting Trials, statement is an important research tool that takes an evidence-based approach to improve the quality of reports of randomized controlled clinical trials. Its critical value to researchers, health care providers, peer reviewers, and journal editors, and health policy makers is the guarantee of integrity in the reported results of research. CONSORT

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[108] Bossuyt, P.M., Reitsma, J.B., Bruns, D.E., Gatsonis, C.A., Glasziou, P.P., Irwig, L.M., Moher, D., Rennie, D., de Vet, H.C., and Lijmer, J.G. (2003). The STARD statement for reporting studies of diagnostic accuracy: Explanation and elaboration. Clin. Chem. 49, 7–18.

[109] Des Jarlais, D.C., Lyles, C., and Crepaz, N. (2004). Improving the reporting quality of nonrandomized evaluations of behavioral and public health interventions: The TREND statement. Am. J. Public Health 94, 361–366.

[110] Altman, D.G., Schulz, K.F., Moher, D., Egger, M., Davidoff, F., Elbourne, D., Gotzsche, P.C., and Lang, T. (2001) The revised CONSORT statement for reporting randomized trials: Explanation and elaboration. Ann. Intern. Med. 134, 663–694.

comprises a checklist and flow diagram to help improve the quality of reports of randomized controlled trials. It offers a standard way for researchers to report trials. The intent is to make the experimental process more clear, flawed or not, so that users of the data can more appropriately evaluate its validity for their purposes.

CONSORT: Checklist of items to include when reporting a randomized trial (RCT)

PAPER SECTION And topic	ITEM	DESCRIPTION
TITLE & ABSTRACT	1	How participants were allocated to interventions (*e.g.*, "random allocation", "randomized", or "randomly assigned").
INTRODUCTION Background	2	Scientific background and explanation of rationale.
METHODS Participants	3	Eligibility criteria for participants and the settings and locations where the data were collected.
Interventions	4	Precise details of the interventions intended for each group and how and when they were actually administered.
Objectives	5	Specific objectives and hypotheses.
Outcomes	6	Clearly defined primary and secondary outcome measures and, when applicable, any methods used to enhance the quality of measurements (*e.g.*, multiple observations, training of assessors).
Sample size	7	How sample size was determined and, when applicable, explanation of any interim analyses and stopping rules.
Randomization --Sequence generation	8	Method used to generate the random allocation sequence, including details of any restrictions (*e.g.*, blocking, stratification)
Randomization Allocation concealment	9	Method used to implement the random allocation sequence (*e.g.*, numbered containers or central telephone), clarifying whether the sequence was concealed until interventions were assigned.

Randomization implementation	10	Who generated the allocation sequence, who enrolled participants, and who assigned participants to their groups.
Blinding (masking)	11	Whether or not participants, those administering the interventions, and those assessing the outcomes were blinded to group assignment. When relevant, how the success of blinding was evaluated.
Statistical methods	12	Statistical methods used to compare groups for primary outcome(s); Methods for additional analyses, such as subgroup analyses and adjusted analyses.
RESULTS Participant flow	13	Flow of participants through each stage (a diagram is strongly recommended). Specifically, for each group report the numbers of participants randomly assigned, receiving intended treatment, completing the study protocol, and analyzed for the primary outcome. Describe protocol deviations from study as planned, together with reasons.
Recruitment	14	Dates defining the periods of recruitment and follow-up.
Baseline data	15	Baseline demographic and clinical characteristics of each group.
Numbers analyzed	16	Number of participants (denominator) in each group included in each analysis and whether the analysis was by "intention-to-treat". State the results in absolute numbers when feasible (*e.g.*, 10/20, not 50%).
Outcomes and estimation	17	For each primary and secondary outcome, a summary of results for each group, and the estimated effect size and its precision (*e.g.*, 95% confidence interval).
Ancillary analyses	18	Address multiplicity by reporting any other analyses performed, including subgroup analyses and adjusted analyses, indicating those pre-specified and those exploratory.
Adverse events	19	All important adverse events or side effects in each intervention group.
DISCUSSION Interpretation	20	Interpretation of the results, taking into account study hypotheses, sources of potential bias or imprecision and the dangers associated with multiplicity of analyses and outcomes.
Generalizability	21	Generalizability (external validity) of the trial findings.
Overall evidence	22	General interpretation of the results in the context of current evidence.

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