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m RNA Purifications Process: Affinity Chromatography, Magnetic Beads and Graphene Coated. Large Scale Production and Toxicological Aspects

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1. Abstract

Aim of this work is to verify the state-of-the-art related m RNA purification process, the technology used as well as the materials employed.

New methods vs classic methods: the use of affinity chromatography or and high-gradient magnetic separation (H.G.M.S.).

In particular the use of magnetic beads since introduction of the graphene coated ones.

Various producers provide many kinds of magnetic beads with various grade of efficiency in separation.

Because today there is a great debate around graphene derivates presence (or not) in vials of m RNA VACCINE it is relevant to better clarify the production process as well and the materials used.

This is of toxicological interest.

2. Keywords: m RNA Vaccine; Production; Purification; Affinity Chromatography; Magnetic Beads Magnetic Beads Graphene Coated; Biopharmaceutical; Large Scale

3. Introduction

During last periods in order to fight Covid-19 pandemic was introduced with emergency authorization a new class of vaccine: The m RNA vaccine.

This innovative biopharmaceutical products were developed starting from the research in the previous decade related oncologic vaccine research.

It is interesting so verifying the manufacturing process used in example to purify this molecule from the impurities or other non-useful component.

The classic method used solvent, but in order to increase efficiency

of the process was introduced purification trough chromatographic methods (in example affinity chromatography and high efficiency methods with high-gradient magnetic separation (H.G.M.S.) and other).

In this process are also used magnetic beads and in order to increase efficiency of interest to verify the role played by the more recent Graphene coated magnetic beads.

Because related the production some m RNA VACCINE it is not officially clarify the complete manufacturing process (probably due to Patents or other industrial secrets) it is crucial to list relevant literature about this argument (for regulatory reason and toxicological aspect).

Various independent researcher reported in some vials of m RNA VACCINE or other covid-19 vaccine particle like graphene inside and various research reported similar particle in blood of vaccinated people.

The fact that using graphene magnetic beads make possible to obtain 170% in efficiency in RNA purification (VS CALSSIC METHODS) is of great interest.

This especially because pharmaceutical producers search great results in production and versus the

Competitors to have more efficient methods is relevant.

In this work are reported a collection of reference involved the keyworks chosen.

All article comes from scientific data base.

In article Journal List Front Bioeng Biotechnol v.7; 2019

Magnetic Separation in Bio-processing Beyond the Analytical Scale: From Biotechnology to the Food Industry

Sebastian P. Schwaminger, Paula Fraga-García, M.Eigenfeld, Thomas M. Becker, and Sonja Berensmeier

"Down-stream processing needs more innovative ideas to advance and overcome current bio-processing challenges. Chromato-graphy is by far the most prevalent technique used by a conservative industrial sector. Chromato-graphy has many advantages but also often represents the most expensive step in a pharmaceutical production process. So, alternative methods as well as further processing strategies are urgently needed. One promising candidate for new developments on a large scale is the magnetic separation, which enables the fast and direct capture of target molecules in fermentation broths. There has been a small revolution in this area in the last 10–20 years and a few papers dealing with the use of magnetic separation in bio-processing examples beyond the analytical scale have been published. Since each target material is purified with a different magnetic separation approach, the comparison of processes is not trivial but would help to understand and improve the magnetic separation and thus making it attractive for the technical scale. We report on the latest achievements in magnetic separation technology and offer an overview of the progress of the capture and separation of bio molecules derived from biotechnology and food technology. The Magnetic separation has great potential for high-throughput down-stream processing in applied life sciences. At the same time, 2 major challenges need to be overcome: (1) the development of a platform for suitable and flexible separation devices and (2) additional investigations of advantageous processing conditions, especially during recovery. Concentration and purification factors need to be improved to pave the way for the broader use of magnetic applications. The innovative combination of magnetic gradients and multipurpose separations will set new magnetic-based trends for large scale downstream processing. Magnetic- separation is an interesting candidate for future down-stream applications due to some important advantageous features:

Integrated 1 step capture and purification of target (high affinity and selectivity)

High throughput

Semi-continuous processing with low energy consumption.

Thus, magnetic separation can help reduce costs and increase yields and productivity compared to traditional processes."

"The webinar will review the basic concepts of magnetic -separation and help the attendees understand how advanced systems may enlight key aspects of the process. These concepts will be applied to parameterize, monitor and validate the magnetic beads behavior in controlled conditions. Afterwards, the discussion will focus on how to transfer the correctly characterized bio-magnetic separation process from laboratory to production scale." (Figure 1).

"Large Scale D.N.A. and RNA Oligonucleotide Production Services

Synthesis: Solid-phase synthesis has been employed for the manufacture of oligonucleotides from micrograms for research use. During the solid-phase synthesis, phosphoramidite monomers are added sequentially onto a solid support to generate the desired full-length oligonucleotide. Each cycle of base addition consists of 4 chemical reactions, detritylation, coupling, oxidation/thiolation, and capping.

Cleavage and Deprotection (C&D): Oligo-nucleotide de-protection involves 3 steps: removal of a cyanoethyl protecting group from the phosphate backbone, cleavage of the oligonucleotide chain from the support, and base deprotection. This process is generally carried out in batch mode where the entirety of the solid support is incubated with C&D reagents.

Purification: The purification of oligonucleotide crude solutions is generally achieved by chromatographic methods. Taking advantage of the internucleotide phosphodiester and phosphorothioate linkage, anion exchanged and ion-paired reversed-phase (IP-RP) chromatographic purifications on HPLC equipment are widely used for the purification of the therapeutic oligonucleotide.

Desalting and Concentration: The most widely accepted choice for therapeutic oligonucleotide isolation at manufacturing scales is the use of tangential flow filtration (TFF). This approach employs an appropriately sized filter membrane and uses a pump to circulate the sample through the TFF set-up making the process more efficient.

Lyophilization: This step is performed using a freezer dryer, which is a machine consisting of a sample chamber, a condenser and a vacuum pump. The oligonucleotide is frozen and lyophilized to obtain the final product in powder form via sublimation." (Figure 2 and 3).

Preactivated Magnetic Beads for Affinity Chromato-graphy

Nhs Mag Sepharose, Sera-Mag Carboxylate-Modified, Sera-Mag Speedbeads Carboxylate-Modifi

"Preactivated magnetic beads are designed for covalent coupling of antibodies, aptamers, and proteins. After coupling is performed, proteins of interest can be affinity captured and enriched using immunoprecipitation (example in Figure reported). The preactivated magnetic beads include NHS Mag Sepharose, Sera-Mag Carboxylate-Modified, and Sera-Mag SpeedBeads Carboxylate-Modified Magnetic Particles".

https://www.cytivalifesciences.com/en/us/solutions/genomics/knowledge-center/magnetic-bead-guide/mag-beads-for-protein-purification

"Magnetic beads are used to purify single proteins, large protein complexes, antibodies and for high-throughput purifications. Magnetic beads based on chromato-graphy resins provide functions of the resin with the convenience and ease-of-use of magnetic beads".

EP2971161A1 European Patent Office

"Ribonucleic acid purification

Disclosed herein are methods for purifying RNA comprising poly A. Also disclosed herein are compositions such as surfaces and oligonucleotides for purifying RNA comprising polyA. Other embodiments are also disclosed. Commercially-available resins having polythymidine oligonucleotide ligands typically contain less than 30 thymidine (2'deoxy) residues and some commercial resin suppliers utilize a distribution of dT chain lengths, not of a discreet length. Commercially-available matrices typically consist of cellulose, latex particles, and magnetic beads containing dT ligands".

Wommer, L.,Soerjawinata, W., Ulber R., Kampeis, P., Agglomeration behaviour of magnetic

microparticles during separation and recycling processes in mR.N.A. purification. Eng. Life Sci. 2021,

"Purification of mR.N.A. with oligo(dT)-functionalized magnetic particles involves a series of magnetic separations for buffer exchange and washing. Magnetic particles interact and agglomerate with each other when a magnetic field is applied, which can result in a decreased total surface area and thus a decreased yield of mR.N.A.. In addition, agglomeration may also be caused by mR.N.A. loading on the magnetic particles. High-gradient magnetic separation (H.G.M.S.) can be used to selectively separate magnetizable components from suspensions. This technique has already been applied by various working groups in the field of biotechnology as well.

It was utilized for the separation of immobilized enzymes and used for the isolation of target molecules. In this process, the target molecule is specifically adsorbed on the functionalized particle surface in a reaction mixture and desorbed again from the magnetic particles (named magnetic beads) after magnetic separation has taken place. An overview of widespread magnetic separators and magnetic particle systems is given in. One widely used micro particle system in the bio separation of proteins, mR.N.A., and viruses is Dynabeads pharmacy is the production of mR.N.A. for vaccine manufacturing.

The synthesis of a mR.N.A. vaccine is described in the literature. Within the production of a vaccine, separation, and purification of mR.N.A. are necessary in the process.

PRACTICAL APPLICATION

Magnetic microparticles have gained importance

in the purification of mR.N.A.-based vaccines. They serve as adsorbents for the mR.N.A.. Through several steps of magnetic separation followed by redispersion of the magnetic beads for washing and elution, the mR.N.A. can be isolated. This is usually done on a mL scale. To obtain larger amounts of mR.N.A., flow-through magnetic separation using high-gradient magnetic separation (H.G.M.S.) can be advantageous. Here, the suspension to be processed is then usually in a stirred feed tank. Due to particle-particle interactions, a not inconsiderable agglomeration may occur, especially due to the attached mR.N.A.. This can reduce the mR.N.A. yield.

For economic reasons it is necessary to perform magnetic particle recycling, for which

there are various process alternatives. With regard to a possible use of H.G.M.S. in an mR.N.A. production for vaccines, particle size distributions were determined to investigate the agglomerating or deagglomerating effect of different process steps"

From Graphene Hydro-gel Could Help mR.N.A. Vaccine Target Cancer More Effectively

Inside Precision Medicine

"A specialized graphene oxide G.O hydro-gel can help stabilize therapeutic mR.N.A. cancer vaccines and release them slowly into the target tissue, show early results from the National Center for Nano-science and Technology in Beijing".

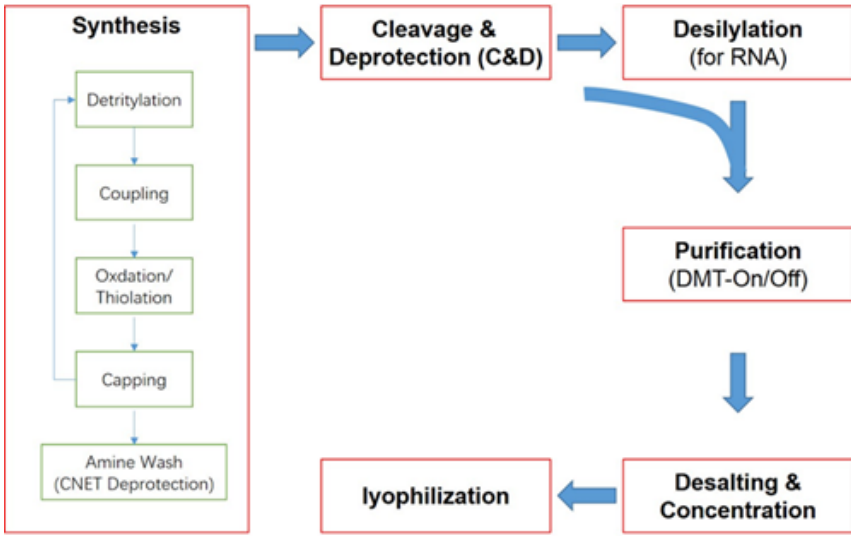

Figure 1: Oligonucleotide production process steps. from https://www.creative-biolabs.com/gene-therapy/large-scale-oligonucleotide-production.htm

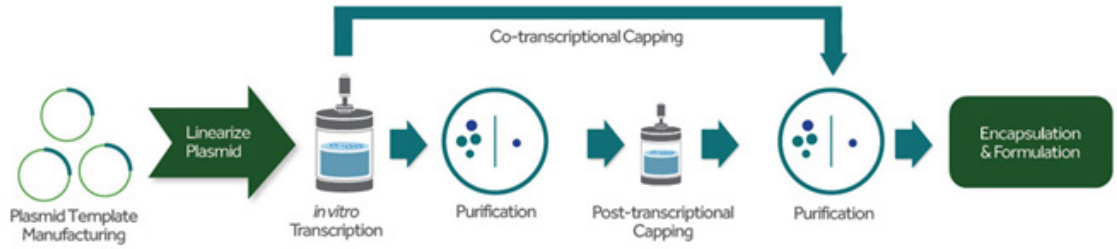

Figure 2: form https://www.codexis.com/

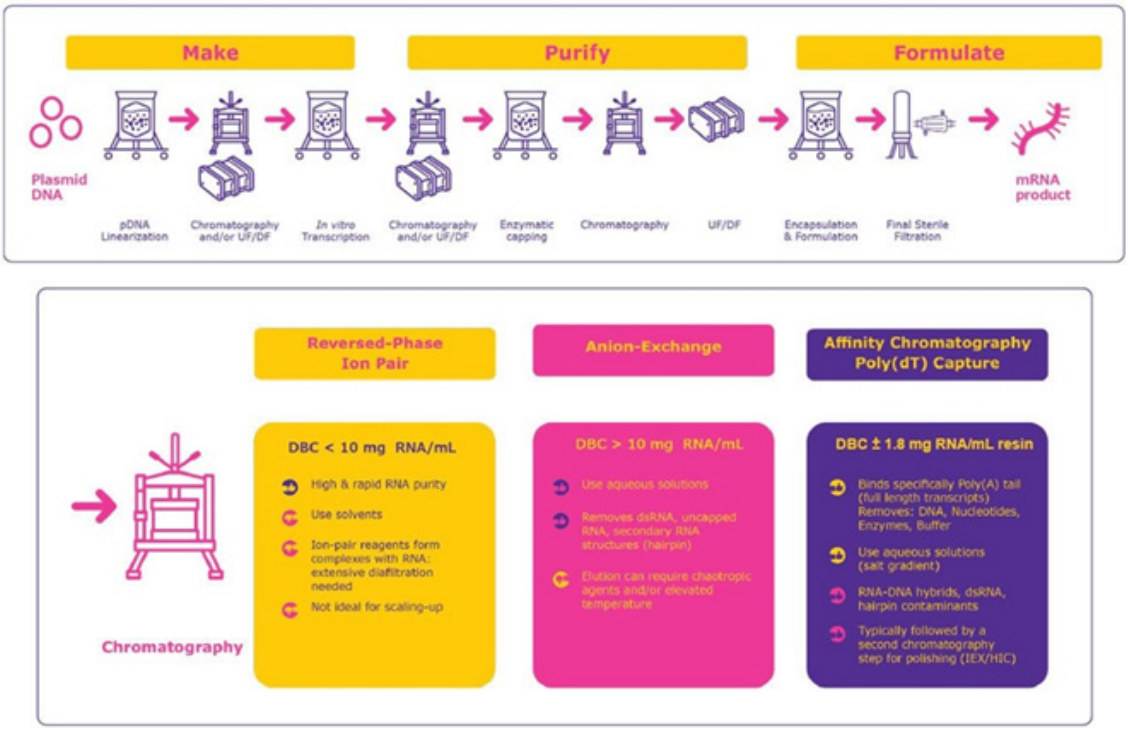

Figure 3: From https://www.sigmaaldrich.com/IT/it/technical-documents/technical-article/pharmaceutical-and-biopharmaceutical-manufacturing/vaccine-manufacturing/manufacturing-strategies-for-mR.N.A.-vaccines

4. Materials and Methods

Related the topics of this work variuos relevant reference are reported, all article comes from PUBMED or other scientific database.

After this review part and ecperimental project hypothesys is submitted to the reseacher in order to provide a global conclusion related to the aim of the work.

5. Results

5.1. From literature

Efficient Lymph Node-Targeted Delivery of Personalized Cancer Vaccines with Reactive Oxygen Species-Inducing Reduced Graphene Oxide Nanosheets

Cheng Xu, Hao Hong, Yonghyun Lee, Kyung Soo Park, M. Sun, Tianrui Wang, Marisa E. Aikins, Yao Xu, and James J. Moon

"Therapeutic cancer vaccines require a -robust cellular immunity for the efficient killing of tumor cells, and recent advances in neo-antigen discovery may provide safe and promising targets for cancer vaccines. Elicitation of T cells with strong antitumor efficacy requires intricate multistep processes that have been difficult to attain with traditional vaccination approaches. A multifunctional nanovaccine platform has been developed for direct delivery of neo-antigens and adjuvants to lymph nodes (LNs) and highly efficient induction of neoantigen-specific T cell responses. A PEGylated reduced graphene oxide nanosheet (R.G.O.-PEG, 20-30 nm in diameter) is a highly modular and biodegradable platform for facile preparation of neoantigen vaccines within 2 h. R.G.O.-PEG exhibits rapid, efficient (15-20% ID/g), and sustained (up to 72 h) accumulation in LNs, achieving >100-fold improvement in LN-targeted delivery, compared with soluble vaccines. R.G.O.-PEG induces intracellular reactive- oxygen species in dendritic cells, guiding antigen processing and presentation to T cells. Importantly, a single injection of R.G.O.-PEG vaccine elicits potent neoantigen-specific T cell responses lasting up to 30 days and eradicates established MC-38 colon carcinoma. Further combination with anti-PD-1 therapy achieved great therapeutic improvements against B16F10 melanoma. R.G.O.-PEG may serve a powerful delivery platform for personalized cancer vaccination." (1)

In Situ Transforming RNA Nanovaccines from Polyethylenimine Functionalized Graphene Oxide Hydro-gel for Durable Cancer Immunotherapy

Yue Yin, Xiaoyang Li, Haixia Ma, Jie Zhang, Di Yu, Ruifang Zhao, S. Yu, Guangjun Nie, and Hai Wang

"Messenger RNA (mR.N.A.) vaccine is a promising candidate in cancer immunotherapy as it can encode tumor-associated antigens with an excellent safety profile. Unfortunately, the inherent instability of RNA and translational efficiency are major limitations of RNA vaccine. We report an injectable hydro-gel formed with graphene oxide (GO) and polyethylenimine (PEI), which can generate mR.N.A. (ovalbumin, a model antigen) and adjuvants (R848)-laden nanovaccines for at least 30 days after subcutaneous injection. The released nanovaccines can protect the mR.N.A. from degradation and confer targeted delivering capacity to lymph nodes. The data show that this transformable hydro-gel can significantly increase the number of antigen-specific CD8+ T cells and subsequently inhibit the tumor growth with only 1 treatment.

This hydro-gel can generate an antigen specific antibody in the serum which in turn prevents the occurrence of metastasis. Collectively, these results demonstrate the potential of the PEI-functionalized GO transformable hydro-gel for effective cancer immuno-therapy."(2)

Nanoscale Research Letters Published: 06 March 2020

Functionalized Folate-Modified Graphene Oxide/PEI siRNA Nanocomplexes for Targeted Ovarian Cancer Gene Therapy

Yunfei Wang, Guoping Sun, Y. Gong, Yuying Zhang, X. Liang & Linqing Yang

Nanoscale Research Letters volume 15, Article number: 57 (2020).

"Gene therapy is emerging as a valid method for the treatment of ovarian cancer, including small interfering RNA (siRNA). Although it is so powerful, few targeting efficient gene delivery systems seriously hindered the development of gene therapy. In this work study, we synthesized a novel gene vector PEG-GO-PEI-FA by functionalized graphene oxide (G.O), in which folic acid can specifically bind to the folate receptor (FR), which is overexpressed in ovarian cancer. Characterizations of the nanocomplexes were evaluated by dynamic light scattering (DLS), atomic force microscopy, and Fourier transform infrared spectroscopy (FTIR). The siRNA condensation ability and stability were assessed by agarose gel electrophoresis. Cellular uptake efficiency and lysosomal escape ability in ovarian cancer cells were investigated by confocal laser scanning microscopy.

Cellular bio safety of the system and inhibitory of the siRNA tolerability were evaluated by CCK-8 assay. The size of the PEG-GO-PEI-FA nanocomplexes was 216.1 ± 2.457 nm, exhibiting mild cytotoxicity in ovarian cancer cells. With high uptake efficiency, PEG-GO-PEI-FA can escape from the lysosome rapidly and release the gene. PEG-GO-PEI-FA/siRNA can effectively inhibit the growth of ovarian cancer cells. By and large, the PEG-GO-PEI-FA/siRNA may offer a promising strategy for siRNA delivery in the treatment of FR-positive ovarian carcinoma or similar tumors" (3)

nature letters article

Graphene as a subnanometre trans-electrode membrane

S. Garaj, W. Hubbard, A. Reina, J. Kong, D. Branton & J. A. Golovchenko

"Isolated, atomically thin conducting membranes of graphite, called graphene, have recently been the subject of intense re-

search with the hope that practical applications in fields ranging from electronics to energy science will emerge. The atomic thinness, stability and electrical sensitivity of graphene motivated us to investigate the potential use of graphene membranes and graphene nanopores to characterize single molecules of D.N.A. in ionic solution" Electrical measurements on graphene membranes in which a single nanopore has been drilled show that the membrane's effective insulating thickness is less than 1 nano-meter. This small effective thickness makes graphene an ideal substrate for very high-resolution, high throughput nanopore-based single molecule detectors (4)

Review Open Access 12 January 2019

Comprehensive Application of Graphene: Emphasis on Biomedical Concerns

S. Syama & P. V. Mohanan

Nano-Micro Letters volume 11

Metrics

"Graphene Substrates for Drug Delivery

GO with its oxygen-containing functional groups (COOH and OH) has been reported as an effective carrier for drug or gene delivery." (5)

J Control Release. 2016 Aug 10 doi: 10.1016/j.jconrel.2016.06.007. Epub 2016 Jun 4.

Efficient mR.N.A. delivery with graphene oxide-polyethylenimine for generation of footprint-free human induced pluripotent stem cells

Hye Yeon Choi, Tae-Jin Lee, Gwang-Mo Yang, Jaesur Oh, J. Won, Jihae Han, Gun-Jae Jeong, Jongpil Kim, Jin-Hoi Kim, B.-Soo Kim, Ssang-Goo Cho

"Clinical applications of induced pluripotent stem cells require development of technologies for the production of "footprint-free" (gene integration-free) iPSCs, which avoid the potential risk of insertional mutagenesis in humans. Previously, several studies have shown that mR.N.A. transfer can generate "footprint-free" iPSCs, but these studies did not use a delivery vehicle and thus repetitive daily transfection was required because of mR.N.A. degradation. We report an mR.N.A. delivery system employing graphene oxide (GO)-polyethylenimine complexes for the efficient generation of "footprint-free" iPSCs. GO-PEI complexes were found to be very effective for loading mR.N.A. of reprogramming transcription factors and protection from mR.N.A. degradation by RNase. Dynamic suspension cultures of G.O-PEI/RNA complexes-treated cells dramatically increased the reprogramming efficiency and successfully generated rat and human iPSCs from adult adipose tissue-derived fibroblasts without repetitive daily transfection. The iPSCs showed all the hallmarks of pluripotent stem cells including expression of pluripotency genes, epigenetic reprogramming, and differentiation into the 3 germ layers. These results demonstrate that mR.N.A. delivery using GO-PEI-RNA complexes can efficiently generate "footprint-free" iPSCs, which may advance the translation of iPSC technology into the clinical settings." (6)

Scanning and Transmission Electron Microscopy Reveals Graphene Oxide in CoV-19 Vaccines

Young RO Published 1 August 2022 Materials ScienceActa Scientific Medical Sciences

"the observations under a pHase Contrast, Dark-Field, BrightField microscopy, Transmission and Scanning Electron microscopy of the vaccine product by Pfizer, including vaccine products of Moderna, Astrazeneca and Janssen revealed some entities that can be graphene strips as seen below in Figure reported. [Figure reported shows an aqueous fraction image from Pfizer vaccine sample (left) and from reduced graphene oxide (R.G.O.) standard (right) (Sigma-777684). Optical microscopy, 100X] [Figure reported Aqueous fraction images containing reduced graphene oxide from Pfizer vaccine sample (left) and sonicated reduced graphene oxide (R.G.O.) standard (right) (Sigma-777684). Optical pHase contrast microscopy, 600X. The Muestra RD1, La Quinta Columna Report, June 28, 2021; Graphene Oxide Detection in Aqueous Suspension; Delgado Martin, Campra Madrid confirmed our findings.]

For a definitive identification of graphene by TEM, it is necessary to complement the observation with the structural characterization by obtaining a characteristic electron diffraction standard sample (as the figure 'b' shown below). The standard sample corresponding to graphite or graphene has a hexagonal symmetry, and generally has several concentric hexagons. [Figure reported Reveals X ray Diffraction Pattern of the Graphene Particles.] Using Transmission Electron Microscopy (TEM) we observed an intricate matrix or mesh of folded translucent flexible R.G.O. sheets with a mixture of darker multilayer agglomerations and lighter colored of unfolded monolayers as seen in Figure reported [Figure reported shows the liposome Capsid containing R.G.O. that Pfizer uses for its product to vehiculate the graphene oxide by attaching the Liposome capsid to specific mR.N.A. molecules for driving the Liposome contents of fGO to specific organs, glands and tissues, namely the ovaries and testes, bone marrow, heart and brain. The image was obtained by a SEM-Cryo preparation.] Using (TEM) revealed an intricate matrix or mesh of folded translucent flexible R.G.O. sheets with a mixture of darker multilayer agglomerations and lighter colored of unfolded monolayers as seen in Figure reported [Fig. reported shows a cluster of graphene nano-particles in a Pfizer vaccine. They appear to be aggregated.] The darker linear areas in Figure reported appear to be local overlap of sheets and local arrangement of individual sheets in parallel to the electron beam. After the mesh, a high density of unidentified rounded and elliptical clear shapes appears, possibly corresponding to holes generated by mechanical forcing of the R.G.O. mesh during treat-

ment as seen in Figure reported. [Figure 4 shows a TEM microscopy observation where particles of reduced graphene oxide in a Pfizer" vaccine" are present. The X-ray diffractometry reveals their nature of crystalline Carbon-based nano-particles of R.G.O.. Energy-Dispersive X-ray Spectroscopy Reveals R.G.O. in Pfizer Vaccine. The Pfizer vaccine liquid fraction was then analyzed for chemical and elemental content using Energy-dispersive X-ray spectroscopy (E.D.S.) as seen in Figure 4. The E.D.S. spectrum showed the presence of Carbon, Oxygen verifying the R.G.O. elements and Sodium and Chloride since the sample shown in Figures reported were diluted in a saline solution. [Figure rported shows an E.D.S. spectrum of a Pfizer "vaccine" under an ESEM microscopy coupled with an E.D.S. x-ray microprobe (X axis =KeV, Y axis = Counts) identifying Carbon, Oxygen, Sodium and Chloride.] The Quantification of mR.N.A. in the Pfizer Vaccine The quantification of RNA in the Pfizer sample was carried out with conventional protocols (Fisher). According to NanoDropTM 2000 spectro photometer calibration check specific software (Thermofisher), the UV absorption spectrum of total aqueous fraction was correlated to 747 ng/ul of unknown absorbing substances. After RNA extraction with commercial kit (Thermofisher), quantification with RNA specific Qbit fluorescence probe (Thermofisher) showed that only 6t ug/ul could be related to the presence of RNA. "(7)

CNIPA china national intellectual property administration

Nano coronavirus recombinant vaccine taking graphene oxide as carrier

"The invention belongs to the field of nano materials and biological medicines, and relates to a vaccine, in particular to development of a 2019-nCoV coronavirus nuclear recombinant nano vaccine. The invention also comprises a preparation method of the vaccine and application of the vaccine in animal experiments. The novel coronavirus vaccine contains graphene oxide, carnosine, CpG and novel coronavirus RBD; The carnosine, the CpG and novel coronavirus RBD are combined on a framework of the graphene oxide; the coding sequence of the CpG is as shown in SEQ ID NO 1; and the novel coronavirus RBDrefers to that a novel coronavirus protein receptor binding region can generate a high-titer specific antibody aiming at the RBD in a mouse body, and strong support is provided for prevention and treatment of the novel coronavirus."(8)

Graphene Oxide Conjugated Magnetic Beads for RNA Extraction

Xuan-Hung Pham , Ahruem Baek , Tae Han Kim , S.Hun Lee , Won-Yeop Rho , Woo-Jae Chung , Dong-Eun

"A magnetic material that consists of silica-coated magnetic beads conjugated with graphene oxide (GO) was successfully prepared for facile ribonucleic acid (RNA) extraction. When the G.O-modified magnetic beads were applied to separate the RNA from the lysed cell, the cellular RNAs were readily adsorbed to and readily desorbed from the surface of the G-O-modified magnetic beads by urea. The amount of RNA extracted by the GO-modified magnetic beads was about 170% as much as those of the control extracted by a conventional phenol-based chaotropic solution. These results demonstrate that the facile method of RNA separation by using G.O-modified magnetic beads as an adsorbent is an efficient and simple way to purify intact cellular RNAs and/or microRNA from cell lysates. Â© 2017 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim". (9)

US20100059449A1

Carbon Coated Magnetic Nano-particles and Their Use in Separation Processes

GOOGLE PATENT

"The invention relates to a process for separating a dispersed phase from a continuous phase comprising the steps of i) contacting said phases with an effective amount of nano-particles; ii) applying a magnetic field gradient to the obtained system; iii) separating the obtained phases wherein said nano-particles are of the core shell type, said core consists of a metal or alloy having soft magnetic properties and said shell contains a graphene layers which are optionally functionalized; to new nano-particles and method of manufacturing such nano-particles. In yet another application e.g. for food additive production or in biotechnical production of proteins, viruses, vaccines and antibodies the magnetic functionalization of the cells may be used to keep them afloat in a desired volume of liquid allowing the separation of the liquid, containing a desired product from their site of production (cells, microorganisms). This application may also be applied in wast-treatment plants."

Development of a 3D-printed single-use separation chamber for use in mR.N.A.-based vaccine production with magnetic microparticles

Lars Wommer,Patrick Meiers, Isabelle Kockler, Roland Ulber, Percy Kampeis

"A new variant of vaccine production is becoming increasingly important with regard to viral infectious diseases. This involves the use of mR.N.A. produced by cell culture methods. This mR.N.A. is protected by special formulations and introduced into human cells, where it induces the expression of proteins and thus, triggers the immune response. In the search for new vaccines, lab. protocols that use functionalized magnetic beads for the purification of mR.N.A. out of cell lysates are often used. For this purpose, magnetic particles with deoxythymidine functionalization (oligo(dT)), with a sequence of 14–25 thymine bases are utilized. Hybridization of the mR.N.A. to the oligo(dT) magnetic particles takes place specifically, due to the base adenine complementary to thymine. Only mR.N.A. molecules have an adenine chain with 40–250 units at the 3′ end. This does not exist on RNA or D.N.A. molecules, so selective sorption occurs by hybridization of mR.N.A. on the oligo(dT) magnetic particles. After separation of the particles loaded with mR.N.A. in the magnetic field and removal of impurities

with various washing steps, elution of the mR.N.A. can be initiated by increasing the temperature. Here, successive multiple magnetic separations and resuspensions are involved, for which suitable millilitre-scale lab. protocols have been developed, some of which are automated.

With the developed SU-H.G.M.S. separation chamber, mR.N.A. manufacture can be realized at such a production scale, which should be sufficient for clinical trials. The standard operating procedures can be transferred directly from the corresponding lab. protocols using magnetic particles. Increasing the amount of magnetic particles to be processed can be easily achieved by parallelization (up-numbering) . High-gradient magnetic separation

If it was possible to use oligo(dT) magnetic particle-based purification of mR.N.A. for clinical trials, there would be no need for a technology change in scale-up from lab. scale to process scale. With high-gradient magnetic separation (H.G.M.S.), the required process technology is available. It has already been used by various working groups in the field of bio technology. The magnetic separators used at H.G.M.S., which operate in the flow-through mode ("magnetic filters"), were and are developed in particular by Franzreb and in own work .

The advantage of this technique in the production of mR.N.A. arises from the fact that the laboratory protocols used by several users in research can be transferred 1:1 to production. (10)"

Fundamentals and Application of Magnetic Particles in Cell Isolation and Enrichment

Brian D. Plouffe, Shashi K. Murthy, and Laura H. Lewis

"Several novel magnetic beads are also currently in development, such as QuickGel™ beads from Quad Technologies (US), new "big beads" from CellCap Technologies Ltd (UK), and metallic beads from TurboBeads Llc (Switzerland). Many of the innovative efforts attempt to challenge the current paradigm of iron oxide beads (50 nm – 10 μm in diameter) that remain attached to the cell surface. QuickGel™ beads are synthesized from a patented hydro-gel technology that allows for facile release of the beads from the cell surface. CellCap beads possess diameters in excess of 50 μm thus their operation relies on gravitational forces combined with magnetic forces to separate labeled cells from suspension. TurboBeads® possess a magnetic metal core and a graphene shell of monolayer thickness; they thus present a high moment and stable labeling potential. Overall, magnetic cell separation is a growing industry and shows much promise for continued future innovations". (11)

Review Vaccine. 2019 Aug 23;37(36):5491-5503. doi: 10.1016/j.vaccine.2018.02.090. Epub 2018 Apr 5.

Affinity chromato-graphy for vaccines manufacturing: Finally ready for prime time?

Mochao Zhao, Melissa Vandersluis , j. Stout , Ulrich Haupts , Matthew Sanders , Renaud Jacquemart

"Although AC has rarely been incorporated in industrial vaccine purification, ion exchange and hydrophobic interaction chromato-graphy (HIC) columns have been widely used in manufacturing-scale processes in combination with other separation techniques such as filtration, precipitation and ultracentrifugation. Affinity chromato-graphy is among the most powerful separation techniques, achieving the finest separation with high yields even in the most challenging feed streams. Incorporating affinity chromato-graphy in vaccine purification has long been attempted by researchers to improve unit yield and purity with the secondary goal of reducing the number of downstream process operations. Despite the success in laboratory-scale proof of concept, implementation of this technique in pilot or cGMP manufacturing has rarely been realised due to technical and economic challenges in design and manufacturing of ideal ligands as well as availability of high-productivity chromato-graphy media. This work paper reviews evolving technologies in engineered ligands and chromato-graphy media that are encouraging companies to re-visit the possible use of affinity chromato-graphy in larger scale vaccine purification. It is postulated that commercial-scale implementation of high throughput single-use affinity chromato-graphy can significantly simplify process architecture, improve productivity and flexibility, and reduce cost of goods. Triple-helix, peptide, and amino acid-D.N.A. affinity chromato-graphy have been studied for pD.N.A. purification. RNA and D.N.A. aptamers have been reported to recognize vaccinia virus, HIV, HCV, and influenza virus " (12) (Figure 4).

Rapid Methods for the Extraction of Nucleic Acids from Biological Samples

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Patent application publication

"The invention is directed to compositions and methods for rapidly and efficiently extracting nucleic acids and/or targeted nucleic acids sequences from biological samples. The methods of the invention comprise combining the sample with a buffer and magnetic silicon beads and concentrating the beads with a magnet or other electrical field. Liquid may be removed, or not, and an alkaline buffer is added followed by magnetic carboxy beads in a binding buffer so that nucleic acids transfer to the carboxy beads, which can be easily and quickly isolated once again with a magnet. Total nucleic acid extraction is greatly enhanced. Extracted nucleic acids can be analyzed, for example, by PCR wherein the nucleic acids can be identified and characterized. Carboxy beads may also contain a ligand so as to target specific nucleic acid sequences. The invention is also directed to kits comprising the tools and compositions for performing the methods of the invention

Exemplary nucleic acid capture matrix (NACM) materials include, preferably, agarose, glass, cellulose, polyacrylamide, Sepharose,

Sephadex, silica, or another matrix media. Preferably, the NACM material is coated with a nucleic acid binding substance (NABS), such as, for example, nucleic acid (NA) binding proteins, antibodies and chemicals with an affinity for NAs including single-stranded nucleic acid sequences. NACM include materials coupled to specific antibodies or antibody fragments or other nucleic acids or ligands that facilitate extract and/or isolation of the diagnostic molecule of interest. Affinity beads are preferably magnetic beads such as, for example, beads commercially available from Dynabeads®, Life Technologies; TurboBeads, TurboBeads Inc. or PureProteome™, and Millipore."

Micromachines Review Magnetic Bead—Magic Bullet

Christine Ruffert

"Nowadays, a huge variety of magnetic beads featuring a large diversity for different applications

is commercially available. Just to name a few: Dynabeads® Magnetic beads provided by Invitrogen,

Estapor® SuperParamagnetic Microspheres and PureProteome™ Magnetic Beads by Merck Millipore,

BcMag™ by Bioclone Inc., ProMag™ and BioMag® from Bangslabs, SupraMag™ by Polymicrospheres

Inc., TurboBeads® by Turbobeads Llc., and SPHERO™ Polystyrene Magnetic Particles by Spherotech.

Other companies like Sigma-Aldrich or Thermo Scientific, Microparticles, and Microspheres-Nanospheres

offer superparamagnetic beads as well. The primary use of these commercial beads is binding,

purification, and magnetic separation of biomolecules comprising proteins, cells, D.N.A. fragments,

and other biomolecules such as nucleic acids, enzymes, antibodies or bacteria". (13) (Figure 5).

Experimental project hypothesis

In order to verify in clear way the productive strategy in m RNA vaccine manifacturing it is needed to receive from the various producers the complete documentation about the purification methods and the characteristics of the material used.

The same this manufacturing process must to be verified by independent professional subjects even if

Regulatory GMP verify was already performed (double check).

In this commission a representative of safety for patient organization must to be included to testify the operations.

Official Documents and photo must to be collected as well as the laboratory chemical analysis related

Impurity (graphene derivates in specific way) using a classic chemical analytical methods whit pre-treatment of the sample (solvent). (16)

It is not acceptable that in official report of assessment it is written that it is needed to complete the information about productive production of an innovative m RNA COVID-19 VACCINE as well as the quality certification of the raw material used.

Timing of the verify: at the start of the production and every 6 month the first year, then 1 time every year

The results of this verify must to be of public availability and uploaded officially in producer's website.

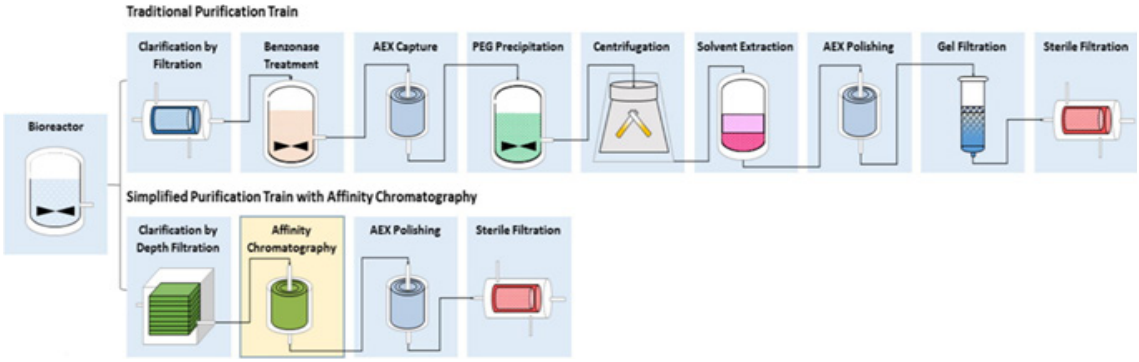

Figure 4: from https://doi.org/10.1016/j.vaccine.2018.02.090

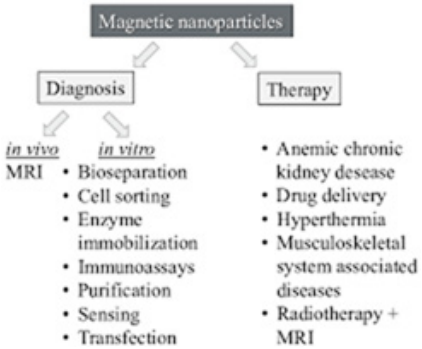

Figure 5: doi:10.3390/mi7020021

6. Discussion

Related the literature reported it is clear that nowadays new methods for purifying RNA are in use

Versus the classic methods.

Large scale production is different vs lab scale.

Between various purifying method: reversed phase ion pair, anion exchange and affinity chromatography is used and with magnetic beads.

Ggraphene modified magnetic beads show great efficacy in this kind of process.

But because m RNA VACCINE manufacturers not clarify in complete way the production process and the toxicological properties of graphene derivated are well known it is crucial to investigate if this new efficient technology is or not used in today production of some covid-19 vaccine.

And related (19 February 2021) EMA/707383/2020 Corr.1*1 Committee for Medicinal Products for Human Use (CHMP)

Assessment report Comirnaty

"Manufacturers

The active substance is manufactured and controlled by either Wyeth BioPharma Division, Andover, United States or by BioNTech Manufacturing GmbH, Mainz, Germany, and Rentschler Biopharma SE, Laupheim, Germany.

During the procedure, a number of issues were highlighted relating to the GMP status of the manufacture of the active substance and of the testing sites of the finished product for the purpose of batch release.

These issues were classified as a Major Objection (MO). After further information was obtained from the sites and inspectors, the MO was considered resolved.

EU GMP certificates for the manufacturing and testing sites were subsequently obtained. In conclusion, appropriate manufacturing authorisations and GMP certificates are in place for all active substance and finished product manufacturing sites. 2 active substance processes have been used during the development; Process 1 and 2. The major changes between AS Process 1 and 2 are: increased process scale, D.N.A. template changed from a PCR template to linearised plasmid D.N.A., magnetic bead purification replaced with proteinase K digestionand UFDF steps.

As regards SO4, the data are requested to be provided regarding the synthetic process and control strategy for the excipient ALC-0315 in order to improve the impurity control strategy, assure comprehensive quality control and batch-to-batch consistency throughout the lifecycle of the finished product

a) A detailed description of the chemical synthesis of ALC-0315 (e.g. information on reagents and process conditions) should be provided. Due date: January 2021

b) Differences in the manufacturing -process between two suppliers should be described and possible impact on impurity profile should be discussed by July 2021. January 2021

c) Information and justification of quality control of starting materials (e.g. general synthetic route, supplier and specifications) and solvents should be provided. Due date: July 2021, Interim report: January 2021

d) Information and justification on critical steps and intermediates (including specifications) should be provided. Due date: July 2021, Interim report: January 2021

e) Specified impurities should be further evaluated and appropriate specification limits for individual impurities should be included when more data are available. Acceptance criteria for specified and un-specified impurities should be added to the specification for ALC-0315 and should also be evaluated during stability studies. Due date: July 2021, Interim report: April 2021

f) The specification limit for total impurities should be re-evaluated as more batch data becomes available and revised, as appropriate. Due date: July 2021

g) The specification limit for assay should be tightened based on the provided batch data to improve the quality control strategy of the finished product. Due date: July 2021

h) Detailed method validation reports for assay, impurities, and residual solvents for ALC-0315 should be provided. Due date: July 2021

i) Results of stability studies in accordance with ICH guidelines should be provided. Due date: July 2021, Interim report: April 2021

7. Conclusion

It is clear that today m RNA VACCINE manufacturing process use non classic methods in purification phases.

Between the more recent technology are also used affinity chromatography and high-gradient magnetic separation (H.G.M.S.).

Magnetic beads are used in this process.

Magnetic beads are produced bay various industry using different technology (13)

Some producers provide also graphene modified magnetic beads to increase efficiency of the process.

For this reason, it is necessary that the vaccine producers provide a complete and full information about the complete manufacturing process as well as the methodology used in purification.

All this related the various technologies in use, the need to get great efficiency in productions and the toxicological implications if impurity of graphene is fined (or not) in final products of vaccine.

In natural way it is possible that producers like to use the really best efficient technologies to get better results in manufacturing

but this must to be linked to the toxicological limits if dangerous substance are used in the purification process.

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