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Review article

mRNA purification: Technology aspects and impurities

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ABSTRACT

mRNA vaccine production like other biopharmaceuticals needs various purification stages in the manufacturing process. The techniques used in this process are including tangential flow filtration (TFF) followed by different chromatographic procedures (affinity and ion exchange separation) with various kinds of resin and then the ultrafiltration-diafiltration (UF/DF) technique. So, it is of interest to verify whether the material used for this kind of procedure is solid phases or the membrane and if released impurity in the final product. From an observational point of view, some relevant literature and references as well as some producers' website and patents were consulted. For silica-based reversed-phase packings, a carbon load percentage indicates the amount of functional bonded phase attached to the silica-base material. This work aims to investigate the role played by these characteristics in the separation process of mRNA. Because this parameter influences the retention time, it is interesting to evaluate the use of the separation technique of biopharmaceuticals and also for the carbon-coated silica columns. Silica gel for chromatography can be produced by synthetic processes as well as from rice husk ash treated at high temperature. This article also describes the effect of using a high level of carbon-coated silica material on the final purified product, impurities occur during the manufacturing process of resins using the graphitic particle and the role of carbon (graphene-quantum dots) membrane reported in various research applications.

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INTRODUCTION

Various technologies are used for the production of mRNA for a vaccine. Production of RNA at a commercial scale does not require cell culture, complex purification or novel equipment. The composition of the in vitro transcription (IVT) reaction is well-defined and a simple proprietary purification process yields more than 99.5% pure RNA (Figure 1). It is a generic, high-yield and disposable process (construct independent) that takes less than eight hours. It yields about 5-6 mg of RNA/mL of IVT reaction which is equivalent to 50-60 human doses. It is assumed that 100µg of RNA is required for a human dose.

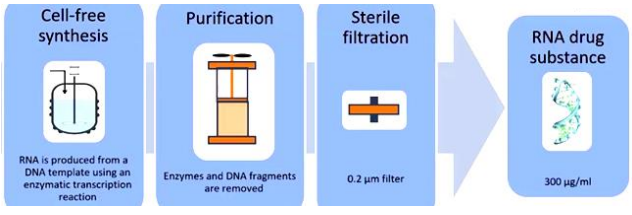

Figure 1. Process of RNA drug preparation

PURIFICATION OF mRNA

The steps for the purification of RNA after its synthesis are including ion exchange chromatography, ultrafiltration (concentration/ diafiltration) and other required processing. Under the IVT step phase, mRNA is purified from impurities and materials used in the previous steps including the endotoxins, and immunogenic double-stranded RNA. (dsRNA), residual DNA template, RNA polymerase and elemental impurities. There are many options available for mRNA purification as given below.

Tangential flow filtration

TFF allows efficient separation of mRNA from smaller impurities that are not retained by the membrane; molecular weight cut-offs ranging from 30 to 300 kDa can be used based on the size of the mRNA. With TFF, it is possible to purify, concentrate and diafilter the product within the same unit operation. At this stage, the mRNA needs to be in the appropriate buffer, either for enzymatic capping or chromatography. An important consideration when using TFF is that the small DNA fragments can

hybridize into the mRNA, generating additional impurities. As seen before, you don't have this risk if using capture to remove the DNA template.

Chromatography techniques

A number of chromatography techniques can be used as an alternative to the TFF and include reverse-phase ion pair, anion exchange and affinity chromatography AC using poly(dT) capture. Chromatography provides an efficient means for DNA template removal/ eliminates the risk of hybridization that can occur during the ultra-/ diafiltration step. It is more expensive and a TFF step would still be required for media exchange and preparation for the subsequent step. Chromatography is also used following the enzymatic capping EC step to remove unwanted products and oligonucleotide impurities coming from the previous enzymatic reaction steps.

Reversed-phase ion pairing

RPIP is commonly used at small scales and allows a very efficient and rapid RNA purification and good separation of single-stranded RNA (ssRNA) from DNA, double-stranded RNA (dsRNA), and short transcripts. This method uses solvents making it poorly suitable for GMP manufacturing production. The technique also requires ion-pair reagents and the resulting formation of complexes with the mRNA may require extensive diafiltration steps for removal. Its sensitivity to fouling by proteins and aggregates makes this kind of technique better suited for polishing than for capture.

Anion exchange

AE has a high dynamic binding capacity and is very efficient for removing immunogenic impurities such as dsRNA, uncapped RNA, RNA–DNA hybrids and other RNA structures. While this allows the use of aqueous solutions, it might require the addition of chaotropic agents that can be toxic and operate at temperatures of up to 85 °C to desorb large mRNA molecules bound to the resin. Ambient temperature TA operations typically elute mRNA species smaller than 500 bases.

Affinity chromatography

AC poly(dT) capture uses a resin to specifically capture the poly(A) tail of full-length mRNA transcripts. This process efficiently removes the DNA, nucleotides, enzymes, buffer components and any other impurities not having a poly(A) tail. The downside of this technique is that, unlike reversed phase and anion exchange, it cannot discriminate dsRNA from ssRNA. In addition, product-related poly(dT) is not efficient for removing other product-related impurities such as DNA fragments that have hybridized into the mRNA. For this kind of reason, the initial chromatography step is AC typically followed by a second chromatography step using an anion exchange for polishing purposes. Following the chromatography step(s), a final concentration and diafiltration are performed to maximize product purity and transfer the mRNA into the appropriate buffer for formulation or storage. At this stage, mRNA can be further purified, concentrated and so diafiltered within the same unit operation. A sterile filtration step can be performed following this TFF step. It should be noted, that sterilizing grade filtration of some mRNAs with a molecular weight of 5000 kDa or higher can be challenging. Of interest, it is to verify the various chromatographic techniques and the material used as well as the chemico-physical process involved.

Column chromatography

Column chromatography is a commonly used purification technique in labs across the world. It can simply and quickly isolate the desired compounds from a mixture. But like many aspects of practical chemistry, the quick and efficient setting up and running of a column is something that can take years to master. Here in this study, we present some of the tips and tricks of the trade to help you set up the perfect column. In a typical column, the stationary phase, a solid adsorbent normally silica gel (SiO_2) or alumina (Al_2O_3), is placed in a vertical glass column. The mobile phase, a liquid, is added to the top of the column and flows down through the column by either gravity or external pressure (flash chromatography, Fig. 2). Separation of compounds is achieved through the varying absorption on and interaction between stationary and mobile phases.

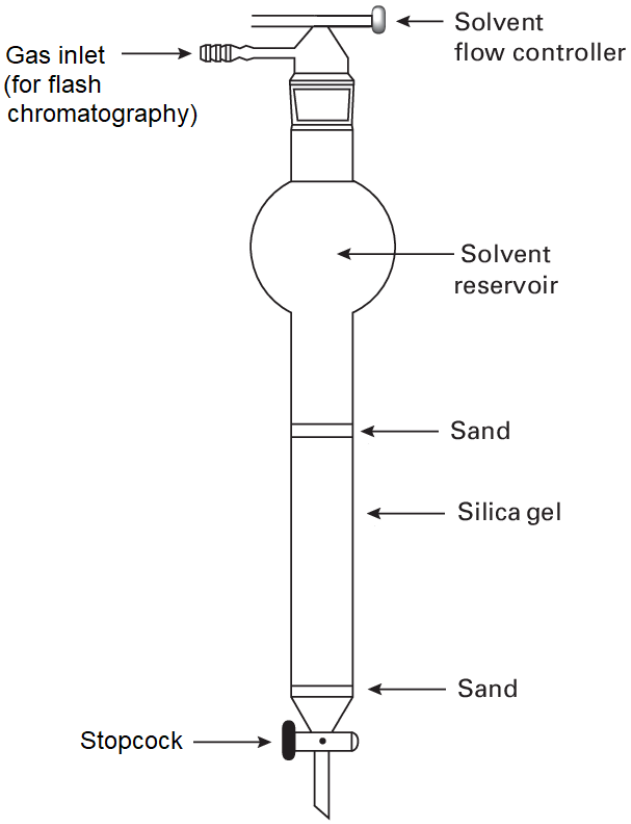

Fig. 2. Apparatus used for a typical flash chromatography

METHOD FOR PRODUCTION OF SILICA GEL

Silica gel (silicon dioxide/ SiO_2) is a chemical composed of silicon and oxygen (SiO_2) and consists of an amorphously structured porous matrix. Silica gel can be produced from the husk of grains, mainly rice.

It is difficult to obtain high-cost silica if high-grade silica is purified from natural ore. It is the current situation. Also, grain shells are not mostly used and are treated as industrial waste at a separate cost. The invention by Zin (2000) has been made in view of such a point, and a high-purity silica gel that can easily be manufactured by using wastes can be easily produced. The purpose is to provide a method of manufacturing the tools. One method for the production of the silica gel comprises burning the husk of grain to provide a burned ash, charging the burned ash in a strongly alkaline aqueous solution containing an alkali metal compound, followed by agitating, to prepare a liquid mixture, heating the mixture and then separating the mixture into ash and a filtrate, steaming the ash with hydrochloric acid to produce activated carbon (AC) as a byproduct, cooling rapidly the filtrate to precipitate a solid, admixing the solid with acid and heating the resultant mixture, separating the mixture into a solid product and a liquid, washing the solid product with water followed by drying to produce high purity (HP) silica gel. The method allows the production of a high-purity silica gel from the husk of a grain which is produced in large quantity and is treated as a waste, with a simple process mainly comprising heating with an aqueous solution and cooling and with ease. The method for producing silica gel according to this invention is as follows: the shell of grain is calcined to form calcined ash, and the calcined ash is added to a water solution containing a strong alkaline component. Is stirred and mixed to prepare a mixed solution, and the mixed solution is heated to a temperature so the solid and liquid are separated into a filtrate and ash. The filtrate collected by solid/liquid separation is cooled. After that, the solid and liquid components are separated into a liquid component and a solid component, and the solid component collected by the solid/liquid separation is washed. Then, the calcined ash obtained by calcining the hull of the substance is stirred and mixed in a water solution containing a strong alkaline component, heated, and then the filtrate obtained by solid-liquid separation is cooled. If the collected solids are washed and then washed, the husks of cereal grains, which are particularly large quantities that become waste, are used as raw materials, heated to an aqueous solution, and cooled by heating. With a simple process, high-purity silica gel can be easily produced (Zin, 2000).

Srivastava et al. (2018) conducted a study on the production of silica gel from rice husk ash (RHA). Different mills in the Bundi district of Rajasthan were visited to know about the production and utilization of rice husk and RHA produced. RHA from different sources was chemically treated and the extracted amounts of silica gel were compared. It was found that RHA contains 70.90% to 84.50% silica gel. This suggests that rice husk (RH), which is considered a waste product from the rice mills and sold at Rs. 300 per quintal, can be used for the production of value-added products such as silica which has its commercial sale value of Rs. 200 per kg. Rice is 1 of the staple foods of India. In Rajasthan, rice is mainly grown in highly rain-fed areas like Kota and Bundi districts. It is estimated that 0.23 tons of rice husk are produced from every ton of rice produced. These mills use 70% of the rice husk produced as fuel in the boiler. Burning RH generates RHA. About 20 million tons of RHA are produced annually in India. The remaining 30% rice husk and RHA, produced by burning rice husk in a boiler, is sold at Rs. 300 per quintal to the poultry to be used as feed, in bricks manufacturing factories, glass industry or used as a bio-fertilizer. On chemical analysis of rice husk ash, it was found that it contains about 80% silica. Silica gel is a non-toxic, non-flammable, non-reactive material. The high surface area of the silica gel crystals, allows it to adsorb water easily, thus making it a useful desiccant. Once saturated with water, the gel can be regenerated by heating it to 120°C for 2 hours. Other uses of silica include its use in column chromatography, insulation powder in steel mills and refrigerators, manufacturing of refractory bricks, and repellents in the form of 'vinegar-tar'. With the increasing grade quality of silica gel, the cost also increases (Srivastava et al., 2018).

Shahnania et al. (2018) produced spherical and porous silica particles from rice husk for chromatographic applications in HPLC columns. After the complete combustion of the rice husk, a white powder of silica was obtained which was dissolved in NaOH and subsequently heated to produce a sodium silicate solution. Spherical porous silica gel was synthesized from prepared sodium silicate in the presence of Pluronic P123 as the surfactant, under an acidic solution. Different porosities were prepared by applying various factors including different vacuums, temperatures and reaction times in order to obtain the optimum conditions for particle ageing. An analytical column was packed with the prepared silica microspheres and evaluated for the separation of 10-deacetylbaccatin III and rutin from taxol and hesperidin, respectively. Rice husk is an agricultural residue which is abundantly produced in the rice industry. Silica is the major element of rice husk ash. Today, some countries must spend high costs to get rid of RH, due to its disposal and environmental problems. Extraction of the silica from RH would be considered a new challenge in order to produce a valuable material from a waste product, and the process is cost-effective because of the high amount of RH as a waste product and the large content of silica in RH ash. There are many reports on the extraction of silica from RH. After complete combustion of RH, approximately 20 wt% of dry RH is ash; the ash itself is consisted of about 90–98% of silica, but the silica content in RHs could be different according to to plant growth conditions. Many approaches to extracting silica from RHs have been investigated. Purification of the obtained silica is crucial for applications as the HPLC stationary phase, and in this favour, various acid leaching procedures have been performed. Kalapathy et al. (2000) scrutinized silica construction using RH as the raw material, dissolved in sodium hydroxide solution. They found that assimilating the initial acid washing of the rice husk ash as well as the final water washing of silica, dramatically intensifies the purity of the silica sample. Following an acid pretreatment step results in highly pure silica which can be used for the preparation of sodium silicate solution via treatment with NaOH. This simple method is based on alkaline extraction followed by acid precipitation to produce pure silica xerogels from RHA, with minimal mineral contaminants. The silica gels produced were heated to 80 °C for 12 h to obtain xerogels. Silica and mineral contents of xerogels were determined by energy-dispersive X-ray and inductively-coupled plasma emission spectrometers, respectively. Xerogels produced from RHA had 93% silica

and 2.6% moisture. The major impurities of the silica produced from RHA at an extraction yield of 91% were Na, K, and Ca. Acid washing prior to extraction resulted in silica with a lower concentration of Ca (<200 ppm). Final water washing of the xerogel was more effective in producing silica with a lower overall mineral content (Na <200 ppm and K <400 ppm). X-ray diffraction patterns revealed the amorphous nature of silica xerogel. Fourier transform infrared data indicated the presence of siloxane and silanol groups. It's worth noting that the mentioned process does not require very high energy, compared to the production of sodium silicate by liquating the quartz and sodium carbonate at a high temperature (1300 °C). Tungkananurak et al. (2007) used the extracted silica from RH for preparation of normal-phase HPLC packing. They developed a simple method to produce mesoporous silica micro-spheres using non-ionic surfactants.

The rice husk production is about 482 million tons per year all over the world. The compounds of the rice husk can be divided into organic and inorganic parts. The organic part contains cellulose, hemicellulose, and lignin and its mineral part consist of silica and metal oxides. One of the most abundant ingredients in the rice husk is silica. 20% of the rice husk is white ash, which is obtained after the complete burning of the rice husk in controlled time and temperatures and is a rich source of silica (more than 90 %). In other countries, this type of recycled silica has many kinds of applications ranging from cosmetics to electronic industries, yet in Iran, recycling of silica does not happen. For the synthesis of spherical porous silica gel from the rice husk by Ghassempour and Shahnani (2019), the physical preparation of the husk was done by burning and acidifying, adjusting the pH, and then heating (burning) at high temperatures to obtain a white powder. So Then, in the alkaline conditions, the powder was converted into a sodium silicate solution. Spherical porous silica gel was produced by the sol-gel method. HPLC columns were prepared by filling the column with bare silica and in the next step, silica was coated with vancomycin. A bare silica column was used for flavonoids analysis and vancomycin coated-silica was tested for the propranolol analysis. The results obtained from the separation of the flavonoids and propranolol showed that the prepared silica could be a very suitable substrate for the settlement of functional groups. The recycling was done successfully and can be used as a column stationary-phase. Various properties that need to be evaluated in column chromatography are given in Fig 3.

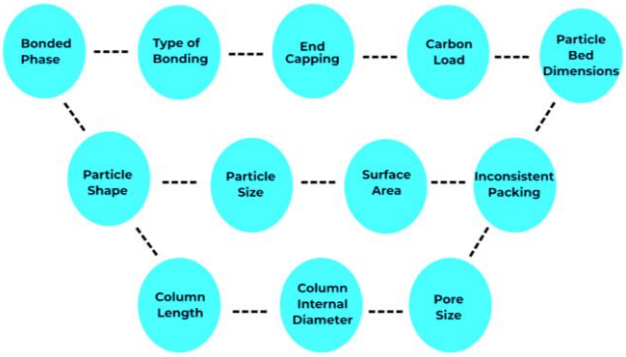

Fig. 3. Properties of stationary phase in column chromatography

CARBON LOAD

Carbon load refers to the percent carbon content of the silica-bonded stationary phase. Generally speaking, a high carbon load (i.e. 18-25%) results in a more hydrophobic surface. The surface is also more resistant to high pH. A high carbon load does not necessarily provide the best resolution. Higher carbon loads generally offer greater resolution and longer run times. Low carbon loads shorten run times and many show a different selectivity, as in Alltech's Platinum line of packings. Hypercard (porous graphitic HPLC column) shows 100% of carbon load.

In the case of reverse phase HPLC, the stationary phase is bonded phase silica columns. An amount of material bonded to the silica is described by the term carbon load which is an amount of carbon as a weight percent of bulk silica packing. For example, the carbon load of the monofunctional C18 column is 7-15% (w/w). The monofunctional column is preferred as it is easier to control and has less batch-to-batch variation. In this case, the higher the carbon load more hydrophobic the column. The stationary phase specifications in HPLC columns as per Alltech Prevail are given in Table 1.

Table 1. Specifications of stationary phase in HPLC columns (BGB Analytik)

Phase	Base material	Particle shape/ size	Pore size/ surface area	Carbon load	Phase type
C18 select	Silica	Spherical/ 3, 5 µm	110 Å/ 350 m^2/g	17%	Monomeric
C18	Silica	Spherical/ 3, 5 µm	110 Å/ 350 m^2/g	15%	Monomeric
C8	Silica	Spherical/ 3, 5 µm	110 Å/ 350 m^2/g	08%	Monomeric
Phenyl	Silica	Spherical/ 3, 5 µm	110 Å/ 350 m^2/g	07%	Monomeric
Cyano	Silica	Spherical/ 3, 5 µm	110 Å/ 350 m^2/g	-	Monomeric
Amino (NH_2)	Silica	Spherical/ 3, 5 µm	110 Å/ 350 m^2/g	-	Monomeric
Silica	Silica	Spherical/ 3, 5 µm	110 Å/ 350 m^2/g	-	-
Organic acid	Silica	Spherical/ 3, 5 µm	110 Å/ 350 m^2/g	-	Monomeric
Carbohydrate ES	Polymer	Spherical/ 5 µm	-	-	-

Based on the above specifications, it is stated that ultra-high purity spherical silica offers excellent stability, efficiency and column-to-column reproducibility. It has stable and strong retention in a 100% aqueous mobile phase for hydrophilic or polar compounds. Due to the high carbon loading, C18 offers a high degree of hydrophobicity and a high surface area offers a high resolution for gradient elution of difficult separation compounds. It is fully endcapped and improves the peak shape. Moreover, the monomeric bonding offers low back pressure and high column efficiency to better resolve chemically similar analytes. A schematic diagram of silica-

carbon composite material as per Xu et al. (2020) is shown in Fig. 4 in which the carrier is silica gel and the carbon layer is of high graphitization degree.

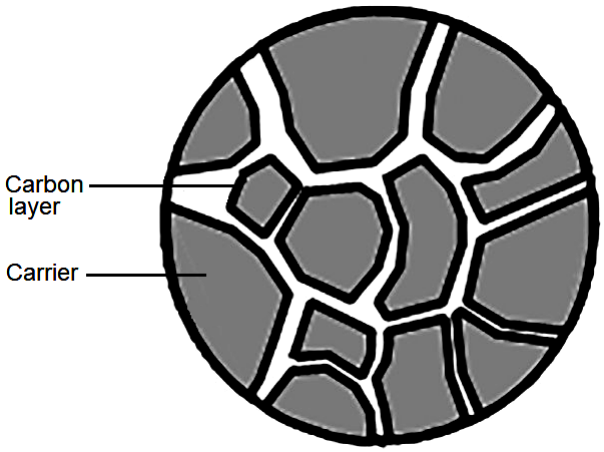

Fig. 4. Schematic diagram of silica-carbon composite material

CARBON LOAD AND SEPARATION – FACTS AND MYTHS

It is usually claimed that the greater the load, the longer the retention in the column. However, most of the time this does not happen. There are many exceptions, and in reality, this rule is false. If the pore size and surface area of the base silica particles are identical, the rule holds. Thus, within a family of packings, the carbon load relationship is true. This guideline lingers as the result of a hold-over from the developing years when there were only a few columns, and many were made from silica of similar density, pore size, and surface area. Predicting retention is a complex task that chromatographers are always attempting to simplify. As a guideline, the carbon load helps in choosing an appropriate packing. With the wide variety of families of chromatographic packings currently available, the use of the carbon load as a guideline is qualitative at best.

There appear to be misconceptions about the role of chain length, carbon loading, surface coverage, and so forth when it comes to the popular alkyl-bonded phases. For a truly reversed-phase mechanism where retention is based upon the relative hydrophobicities of analyte molecules, retention is usually based upon the carbon load.

Carbon load can be proportional to the chain length but not necessarily so. A typical silica gel has about 8.0 μmol/m^2 of reactive silanols used for bonding organosilane reagents. If, for a given surface coverage of a monolayer bonded phase in μmol/m^2, the longer the chain length, the more carbon would be present and retention would be proportional to the chain length. If a shorter chain bonded phase (say, a C8) had a higher surface coverage resulting in more carbon on the surface, then it could have more retention than a C18 bonded phase. According to Thermo Scientific, Hypercarb Porous Graphitic Carbon HPLC columns offer 100% porous graphitic carbon for extended separation capabilities. It shows the enhanced retention of polar compounds and separation of structurally related analytes with stability at pH extremes and high temperature.

CHARACTERISTICS OF STATIONARY PHASE SUPPORT

The two main aspects of the chemical characteristics of solid support, which are of interest, include 1) its internal chemical composition and 2) the chemical functionalities allowing the binding of the active phase (in cases when the solid support does not act itself as the active phase). Regarding the internal chemical composition, the solid support can be made from silica, ethylene/ propylene bridged silica, hydrated zirconia, hydrated alumina, aluminosilicates, porous graphitic carbon, zeolites, or various organic polymers such as polystyrene crosslinked with divinylbenzene (PS-DVB), methacrylates, etc. Recently, metal-organic frameworks were experimentally evaluated as support for HPLC stationary phases. Regarding the other materials, columns based on hydrated zirconia are commercially available, but, they have lower chromatographic performance compared with those based on silica mainly due to numerous Lewis acid sites present on the stationary phase. Commercially available are also the porous graphitic carbon columns. In order to achieve a large surface area, graphitic stationary phases are made using silica as a template on which a layer of organic material is applied followed by pyrolysis in an inert atmosphere to generate graphite. This is followed by the dissolution of the silica template (Moldoveanu and David, 2022).

This type of column has a strong hydrophobic character, but some problems with surface homogeneity remain to be solved. For the preparation of hydrophobic phases with the use of vertical polymerization, various levels of carbon load (C%) can be placed on the silica surface, C% varying depending on the procedure between 5% and 30%.owever, even for columns containing the same type of phase, like as C18, many variations in the active phase structure are possible. The variations may include the type of bonding (mono-, di-, tri-functional), the type of polymerization (horizontal, vertical), the carbon load, the density and uniformity of the coverage of solid support (of silica), and the variations in endcapping.

PREPARATION OF CARBON-COATED SILICA

Carr et al. (2011) invented a method using the homogeneous precipitation of a metal on a surface of a particle to prepare silica particles having the metal adsorbed thereon. In certain embodiments, the silica particles having the metal adsorbed thereon can be used to prepare the carbon-coated silica particles. The carbon-coated silica particles can be useful in a wide variety of applications including, for example, for use as sorbents in chromatography. Carbonaceous materials are versatile sorbents used in a wide range of applications, most particularly, for gas-liquid chromatography. Commercial carbon phases for LC, carbon clad zirconia (C/ZrO_2) and the porous graphitic carbon (Hypercarb) among all the available reversed-phase materials show unique forms of chromatographic selectivity for polar and nonpolar compounds, as well as for structural isomers, and thus have been used to separate the analytes that are not readily resolved by conventional reversed phases (like, alkyl silica phases). Neither substrate gave an absolutely uniform carbon deposition; both require much more than a

theoretical mono-layer of carbon to achieve maximum retentivity. The percent carbon required to form one monolayer is about 7% (w/w). About 32% and 25% of carbon are needed for the low and high levels of Al(III) treatments of silica respectively to obtain maximum retentivity and to fully sequester the Al(III) sites on the silica. These carbon loads correspond to about 4-5 carbon mono-layers which strongly suggests that carbon deposition is non-homogeneous. It is believed that carbon deposition does not proceed monolayer by monolayer, which is, in fact, commonly observed from the deposition of the pyrolytic carbon. In some embodiments, the chemical vapour deposition process includes contacting the silica particles having the metal adsorbed thereon with an organic vapour under conditions effective to form the carbon-coated silica particles. In some kind of embodiments, the organic vapour includes one or more hydrocarbons (one or more C1-C12 hydrocarbons such as hexane). Exemplary conditions effective to form the carbon-coated silica particles include a temperature of about 500 °C, in some embodiments a temperature of at least 600 °C, and in other embodiments a temperature of at least 700 °C. Carbon-coated silica particles prepared by such methods are also disclosed, and the disclosed methods may also include separating and/or drying the carbon-coated silica particles. In another aspect, this specification provides a carbon-coated silica particle including a silica particle and monolayers or less (in some embodiments, one monolayer or less) of Al(III) cations on the surface of the silica particle; and a layer of carbon deposited over Al(III) cations on the surface of the silica particle, wherein the carbon-coated silica particle includes 15-50 wt% carbon. In some embodiments, the carbon-coated silica particle includes 20-40 wt% carbon, and in certain embodiments 25-35 wt% carbon. Carbon was deposited on both quarter-and full-monolayer Al(III) treated silica, and the carbon load was adjusted by varying the reaction time. Both substrates showed increases in carbon load with time, but the increase is much faster with a full monolayer of Al(III). As per the TOSOH catalogue, the TSKgel G-DNA-PW column is dedicated to the separation of large polynucleotides, such as DNA and RNA fragments of 500-5,000 base pairs. TSKgel ODS-100 is the first choice when a universal reversed-phase column is needed for two levels of hydrophobicity (15% and 20% carbon load).

To make the carbon phase on silica, Paek et al. (2011) treated the silica surface with a monolayer or less of metal cations that bind to deprotonated silanols to provide catalytic sites for carbon deposition. After Al(III) treatment, a carbon phase is formed on the silica surface by chemical vapour deposition at 700 °C using hexane as the carbon source. The amount of Al (III) on the surface was varied to assess its effect on carbon deposition, and the carbon loading was varied at different Al (III) levels to assess its effect on the chromatographic properties of the various carbon adsorbents. To prepare metal-adsorbed silica, Aluminum chloride hexahydrate was used for the Al(III) treatment. Silica, 13.7 µm AstroSil was used for the preliminary CVD study with Al(III) metal treatments, and 5 µm Zorbax silica was used to prepare HPLC supports with Al(III) treatment. For comparison, attempts were also made with Leboda's choice of Zr(IV) using zirconium tetrachloride, but Al(III) was much more effective for carbon deposition.

Nano-silica extracted from rice husk and its application in acetic acid steam reforming was studied by Guo et al. (2021). A simple and efficient heat treatment method was used to extract high specific surface area silica from rice husk. It was found that the acid-leaching process was beneficial for the removal of metal impurities and the decomposition of organic substances. The carbon residue decreased and sample purity increased with increasing temperature. At 600 °C, silica with a yield of 21.7% and a purity of 99.45% was obtained. As seen in Fig. 5, it is obvious that the pyrolysis of organic matter is incomplete at low temperatures. An element analyzer was used to analyze the carbon residue in the product. As the temperature increases, the residual carbon content decreases. When the temperature increases from 400 °C to 500 °C, the residual carbon content decreases sharply from 9.13% to 0.19%. When the temperature continues to rise, the residual carbon reduction rate slows down. It indicates that the pyrolysis is insufficient at a temperature lower than 500 °C, leading to a large amount of remaining organic matter in the sample which appears black at 400 °C. When the pyrolysis temperature is 500 °C, the sample appears light yellow with a small amount of remaining organic matter. When the temperature rises to 600 °C, the residual carbon content reduces to 0.09% with the sample purity increase. The 600 °C sample appears white, indicating that the pyrolysis is sufficient at the temperature. When the pyrolysis temperature is continuously increased, the residual carbon content is further reduced to 0.01%.

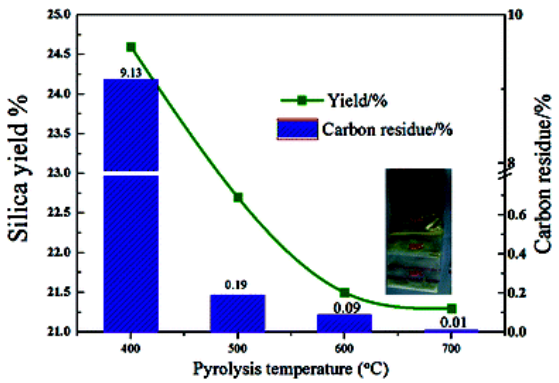

Fig. 5. Change of rice husk pyrolysis residual carbon content with temperature [silica yield and carbon residue content of rice husk with 8 wt% acid leaching pretreatment, pyrolysis changes with temperature]

As per Shen et al. (2014), a schematic diagram for the preparation of adsorbents, carbon and silicon-derived materials from rice husk ash char is given in Fig. 6.

Silica from rice husk ash Amorphous silica powder is a basic raw material used in industries associated with rubber, ceramics, electronics, catalysis, pharmaceutics, dental material and other materials. When rice husk is burnt in the air, it leads to the formation of silica ash, which varies from grey to black depending upon inorganic impurities and unburnt carbon amount. RHA can be obtained by burning rice husk in an electric furnace at 600 °C for 4 hours (Prasad and Pandey, 2012).

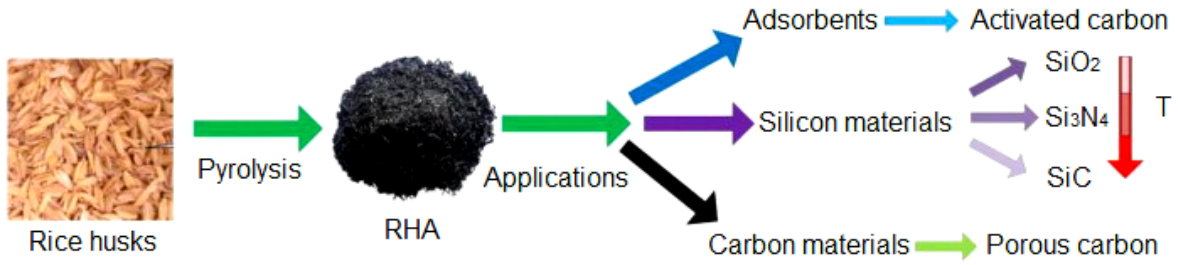

Fig. 6. Different steps for preparation of various materials from rice husk char

Mayadunne and El Rassi (2014) suggested two approaches for incorporating carbon nanotubes into monolithic columns for HPLC. They pertain to the investigation of carbon nanotubes either (i) as entities to modulate solute retention on monolithic columns bearing well-defined retentive ligands or (ii) as entities that constitute the stationary phase responsible for solute retention and separation. Approach (i) involved the incorporation of carbon nanotubes into octadecyl monolithic columns while approach (ii) concerns the preparation and evaluation of ideal monolithic support and coating it with carbon nano-tubes to yield a real "carbon nanotube stationary phase" for the HPLC separation of a wide range of solutes. First, an octadecyl monolithic column based on the in situ polymerization of octadecyl acrylate and trimethylolpropane trimethacrylate was optimized for use in HPLC separations of small and large solutes (proteins). To further modulate the retention and separation of proteins, small amounts of carbon nanotubes were incorporated into the octadecyl monolith column. In approach (ii), an inert, relatively polar monolith based on the in situ polymerization of glyceryl monomethacrylate (GMM) and ethylene glycol dimethacrylate (EDMA) proved to be the most suitable support for the preparation of carbon nanotube stationary phase. This carbon nanotube-coated monolith proved useful in the HPLC separation of a wide range of small solutes including enantiomers. In approach, more homogeneous incorporation of carbon nanotubes into the diol monolithic columns (GMM/EDMA) was achieved when hydroxyl functionalized carbon nanotubes were incorporated into the GMM/EDMA monolithic support. In addition, high-power sonication for a short time enhanced further the homogeneity of the monolith incorporated with nanotubes. In all cases, nonpolar and π interactions were responsible for solute retention on the monolith-incorporated carbon nanotubes.

A US-based producer of magnetic beads for RNA separation is used TFF filtration for the separation of DNA and RNA. Although TFF filtration can separate RNA from DNA, however, the process is inefficient. The limitations are that it cannot separate double-stranded RNA (dsRNA) from single-stranded RNA (ssRNA). dsRNA is a contaminant which lowers the efficacy of the vaccine. So there is a secondary column filtration to remove dsRNA. Also, the TFF filters need to be replaced often. Over time, the TFF filters become lubricated with lipids and DNA contamination starts to slip through. A better process can be developed where the carbon pulls off only the RNA and leaves the DNA behind using Ca^{2+} as the binding agent. The translation reaction can happen in the presence of 100 mM Ca^{2+} so this would allow RNA to continuously be removed in the process. The magnetic beads are then isolated and the RNA is released. They use polysulfone membranes for secondary columns. There is no resin, it is a filter. It is a plastic filter with holes in the walls of a specific size. Separation of dsRNA and ssRNA is done with silica columns. Separation of RNA and DNA is done with a polysulfone size filter, but the polysulfone is not reactive in any way, it is simply plastic with holes of a specific size.

COMMERCIAL SILICA

Silica comes in different qualities. The cheapest silica is refined sand or silica from a rice hull. Cheap silica is harvested from the natural environment. This type of silica likely contains carbon. This is not what is used in sensitive applications. Synthetic silica gel, the most expensive silica, is reduced from sodium silicate and $CoCl_4$. There is no carbon in the reaction which produces it. The pharmaceutical industry only uses synthetic silica gel. So there is no carbon. Some producers coated artificially with graphene the silica particle to vary the retention time. There are problems separating ssRNA and dsRNA. Also, the TFF filters are very expensive and need to be replaced constantly. This carbon load problem has never been mentioned and these are bigger concerns right now in manufacturing.

As per Sigma Aldrich, there are at least 50 types of silica gel. Also, some silica is artificially coated with carbon polymers. Doing this changes the hydrophilic or hydrophobic characteristics. This type of silica is generally very expensive and not often used in industry. It is more common in research labs. The cheapest silica can come from the shells of diatoms, and shells of algae. Some more refined silica can come from rice hull ash or other similar sources. The best and most expensive silica is fully synthetic. This does not cover all the types. There are many other types. All pharmaceutical applications use synthetic silica. Other types of silica are generally only used in chemical production. Silica separates compounds based on the retention time of the molecules. Silica is polar and non-polar molecules move more quickly through the silica. It is based on interaction with the silica. Stronger interaction leads to slower movement through the silica column. The C8 or C18 groups are added to make silica less polar. This is useful when you are separating compounds that are both non-polar. These would normally both move very quickly through silica. The C8 or C18 groups slow them down which leads to better separation. These groups are intentionally added to specific types of silica. When separating RNA, there is no need to do this. There is no carbon load in synthetic silica gel the load is

0%. On Sigma Aldrich as well and for rice hull ash silica, the carbon load is about 3%. This would not be used in synthetic RNA production. The application is too sensitive.

RNA PURIFICATION TO PREPARE VACCINES

Joseph et al. (2014) disclosed the methods for purifying RNA comprising poly-A. Also disclosed compositions such as surfaces and oligonucleotides for purifying RNA comprising poly A. 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. These compositions and methods can use a surface linked to poly T/U. The term surface refers to a part of a support structure (e.g., a substrate) that is accessible to contact with one or more reagents, poly T/U oligonucleotides, etc. The shape, form, materials, and modifications of the surface can be selected from a range of options depending on the application. In one embodiment, the surface is sepharose. In one embodiment, the surface is agarose. The surface can be substantially flat or planar. Alternatively, the surface can be rounded or contoured. Exemplary contours that can be included on a surface are wells, depressions, pillars, ridges, channels or the like. Exemplary materials that can be used as a surface include, but are not limited to acrylics, carbon, cellulose, ceramics, controlled-pore glass, cross-linked polysaccharides, gels, glass, gold, graphite, inorganic glasses, inorganic polymers, latex, metal oxides, metalloids, metals, mica, molybdenum sulfides, nanomaterials, nitrocellulose, nylon, optical fibre bundles, organic polymers, paper, plastics, polacryloylmorpholide, poly(4-methylbutene), polyethylene terephthalate, poly(vinyl butyrate), poly-butylene, polydimethylsiloxane, polyethylene, poly-formaldehyde, polymethacrylate, polypropylene, poly-saccharides, polystyrene, polyurethanes, polyvinylidene difluoride, quartz, rayon, resins, rubbers, semiconductor material, silica, silicon, sulfide and teflon.

The pharmaceutical industry employs Aerosil 200V as an excipient in various formulations in a range of concentrations from 0.5 to 20%. Ledesma et al. (2015) evaluated the behaviour of silicon dioxide obtained from rice husk as a substitute for Aerosil 200V in the formulation of Valsartan 160 mg tablets. This new material was characterized and recognized for its physical, mechanical and technological properties. Three pilot batches of tablets were manufactured and evaluated employing the rice husk ash and Aerosil 200V. Stability studies were done for 6 months including moisture influence during the storage period. The rice husk ash meets established quality specifications in the pharmacopoeia USP and BP to colloidal silicon dioxide. At zero and six months the analyzed parameters were within the ranges established in the manufactured tablets with the two materials. It is possible to use silicon dioxide obtained from rice husk as an excipient in the Valsartan 160 mg tablets formulation as a substitute for Aerosil 200V and so decreasing agro-industrial waste. The silicon dioxide obtained from rice husk presented similar characteristics to the Aerosil 200V (colloidal silicon dioxide). Its analytical quality was according to the requirements of the BP2011 and USP34 pharmacopoeias.

mRNA VACCINES

Recently Moderna and BioNTech developed the most successful mRNA vaccines for COVID-19. There are some basic differences between Moderna mRNA-1273 and Pfizer-BioNTech BNT162b2 COVID-19 mRNA Vaccines (Ouranidis et al., 2022). These were the most thoroughly tested, widely employed and successful mRNA products in the market developed from liponanoparticle mRNAs. The Moderna mRNA product contains a 4004-nucleotide sequence encoding for the spike protein of the virus, whose features were not disclosed by the company but were retrieved through means of reverse engineering. It features 2 serial proline substitutions at 986 and 987 amino acid sites alongside the presence of the furin cleavage site, modifications that code for a stable prefusion S viral protein. It is 50-capped utilizing the cap 1 technology and its 50 untranslated regions are thought to be a patented V1-UTR.

The main coding sequence is characterized by the substitution of uridine with N1-methylpseudouridine and codon sequence optimization that substitutes all GAA codons with GAG. As a 30 untranslated region, Moderna employs the one located on the human β-globin gene and terminates the sequence using 3-stop codons, while the poly-A tail remains to be determined. The liponanoparticles contain 1,2- dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000 (PEG2000-DMG), Heptadecan9-yl 8-((2-hydroxyethyl)(8-(nonyloxy)− 8-oxooctyl)amino)octanoate) (SM-102), cholesterol and 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC).

Excipients contained within the final carrier preparation include acetic acid, tromethamine and its hydrochloride salt, sucrose and sodium acetate. It is dosed as 100 µg µRNA. The mRNA of the BioNTech product has a length of 4284 nucleotides and it encodes the spike protein of the virus as well. Like the Moderna vaccine, it features the same two serial proline substitutions but omits the furin cleavage site, resulting in a similar stable prefusion S protein. It is 50 capped with a modified cap 1 analogue and the 50 untranslated regions are comprised of a fragment of the human α-globin gene. The main coding sequence employs the same uridine substitutions for the modified analogues but compared to Moderna, utilises a more lenient approach as far as GAA codons are concerned, leaving 14 of them unchanged. The 30 untranslated region of the construct features a hybrid sequence comprised of Amino-terminal enhancer of split gene sequence and mtRNR1 mitochondrial 12S ribosomal RNA 30 regions, while it terminates with 2 UGA stop codons and features a segmented poly-A tail.

Nanoliposomes are formed utilizing DSPC, (4-hydroxybutyl)azanediyl)bis(hexane-6,1-diyl)bis(2-hexyldecanoate) (ALC-0315), cholesterol, and 2[(polyethylene glycol)-2000]-N,N-ditetradecylacetamide, while the excipients include potassium chloride, sodium chloride, sucrose, monobasic potassium phosphate and dibasic sodium phosphate dehydrate. The finished product has a dosage of 30 µg mRNA. Purification of the linearized pDNA template ensues, usually by means of silica columns or chromatography, prior to its use as an in-vitro transcription template (Ouranidis et al., 2022). Different strategies for developing mRNA vaccines are given in Table 2.

Table 2. Strategies for developing mRNA vaccines

Vaccine	IVT pol	5'-cap	Codon optimization	Antigen design	Modified nucleotide	Purification method
mRNA-1273	T7	M7GpppNmN	Yes	Full length S protein K986P/V987P	N1-methyl pseudouridine	Oligo-dT
BNT162b (3 LNP-mRNAs)	T7	M7GpppNmN	Yes	S protein RBD subuit K986P/V987P	N1-methyl pseudouridine	Magnetic purification
CVnCoV	T7	M7GpppNmN	Yes	Full length S protein K986P/V987P	N1-methyl pseudouridine	LiCl precipitation
LUNAR-COV19	T7	M7GpppNmN	Yes	VEEV-FL-S protein	N1-methyl pseudouridine	Silicon column
LNP-nCoVsaRNA	T7	M7GpppNmN	Unknown	VEEV-FL-S protein	Unknown	LiCl precipitation

RECOMMENDATION

For investigating the role played by the carbon load of the silica separation process on the mRNA purification process, it is necessary to test two kinds of columns, 1) low carbon load and 2) high carbon load (graphitic) using different silica gel either from synthetic origin or from rice husk (burned at high temperature). After this separation process, it is needed to test the amount and quality of the mRNA extracted and the presence or absence of graphene-graphitic impurity in the final product (vials). These entire tests must be performed by an independent official certified chemical laboratory. A difference insignificative way ($p > 0.05$) in the results imply to take into consideration; this aspect to find more safety purification process. The results must be sent to the concerned regulatory agency.

DISCUSSION

Silica particles for separation are generally synthesized and so usually this product does not contain carbonaceous particles, but sometimes these particles are artificially coated whit graphene to increase efficiency. Another way some producers indicate the production of silica gel from rice (is a simple method for the production of pure silica from rice hull ash. Various producers can provide silica columns for the separation of biopharmaceuticals with different levels of carbon load. These properties indicate the % amount of bonded material to the silica particle. Some silica particles for chromatographic use are carbon-coated to modify their chemico-physical properties. Graphene derivatives are also used for example in some magnetic beads to increase the efficacy of separation. In the literature are reported various research related to carbonaceous product use in research, laboratory or for testing need to purify RNA. Because today there is a great public debate related to the findings by some independent researchers of graphene-like particles in some vials of covid-19 vaccine as well as in the blood of vaccinated is crucial to more deeply investigate the role played by carbon product silica materials if used in large scale purification of RNA or not.

CONCLUSION

Because mRNA vaccine producers not full have clarified the production processes as well as the material used it is interesting to submit to the researcher the fact that in the separation-purification of RNA, various techniques were and are used (first used membrane, the magnetic beads since monoliths today). mRNA vaccines are purified using TFF combined with a silica chromatographic separation or other resin by magnetic methods. This kind of resin can also be carbon coated (research scale-testing).

Exemplary materials that can be used as a surface include, but are not limited to acrylics, and carbon (e.g., graphite, carbon fibre). If silica gel is used, this is produced artificially synthesized or comes from environmental products like rice and others. The silica gel synthetically produced is more expensive than the one produced from rice. The syntactical ones are used especially in research, silica from rice is not used for sensitive applications.

After TFF and affinity or ion chromatographic separation, there is a phase of ultrafiltration/diafiltration that uses various kinds of the membrane. Graphene and carbon material are used in some separative procedures (especially in other fields like water purification). Various independent researchers found graphene-like a particle in some covid-19 vaccine vials. The producers of this vaccine did not fully clarify the purification process and the material used is crucial to investigate the role played by this characteristic (carbon load in silica separation process) in the final product impurity profile. A specific toxicological and regulatory public interest requires a more deep investigation of the role played by graphene carbon compounds in the purification of the resin used in RNA biopharmaceuticals.

CONFLICTS OF INTEREST

The author(s) declare(s) no conflicts of interest.

DECLARATION

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REFERENCES

Carr PW, Mccormick AV, Paek C (2011). Carbon coated silica particles and methods of making same. Patent No. WO2011150179A2 WIPO (PCT). Available at: https://patents.google.com/patent/WO2011150179A2/en

Ghassempour A, Shahnani M (2019). Recycling silica from rice husk and its application in liquid chromatography. Environmental Sciences, 16(4), 81-92.

Guo W, Li G, Zheng Y, Li K (2021). Nano-silica extracted from rice husk and its application in acetic acid steam reforming. RSC Advances, 11, 34915-34922. https://doi.org/10.1039/D1RA05255A PMid:35494779 PMCid:PMC9043021

Joseph W, Grant J, Bancel S (2014). Ribonucleic acid purification. Patent No. WO2014152031A1 WIPO (PCT). Available at: https://patents.google.com/patent/WO2014152031A1/en

Kalapathya U, Proctora A, Shultz J (2000). A simple method for production of pure silica from rice hull ash. Bioresource Technology, 73(3), 257-262. https://doi.org/10.1016/S0960-8524(99)00127-3

Ledesma EF, Acosta CR, Garrido ML, Polanco ID, Guarnaluce DC (2015). Evaluation of rice husk as an excipient for the pharmaceutical industry. Journal of Materials and Environmental Science, 6(1), 114-118.

Mayadunne E, El Rassi Z (2014). Facile preparation of octadecyl monoliths with incorporated carbon nanotubes and neutral monoliths with coated carbon nanotubes stationary phases for HPLC of small and large molecules by hydrophobic and π-π interactions. Talanta, 129, 565-574. https://doi.org/10.1016/j.talanta.2014.06.032 PMid:25127634 PMCid:PMC4134917

Moldoveanu SC, David V (2022). Progress in Technology of the Chromatographic Columns in HPLC. In S. C. Moldoveanu, & V. David (Eds.), Analytical Liquid Chromatography - New Perspectives. IntechOpen. https://doi.org/10.5772/intechopen.104123

Ouranidis A, Vavilis T, Mandala E, Davidopoulou C, Stamoula E, Markopoulou CK, Karagianni A Kachrimanis, K (2022). mRNA Therapeutic Modalities Design, Formulation and Manufacturing under Pharma 4.0 Principles. Biomedicines, 10, 50. https://doi.org/10.3390/biomedicines10010050 PMid:35052730 PMCid:PMC8773365

Paek C, McCormick AV, Carr PW (2011). New method for development of carbon clad silica phases for liquid chromatography: Part I. Preparation of carbon phases. Journal of Chromatography A, 1218(10), 1359-1366. https://doi.org/10.1016/j.chroma.2010.12.114 PMid:21295308 PMCid:PMC3205950

Prasad R, Pandey M (2012). Rice Husk Ash as a Renewable Source for the Production of Value Added Silica Gel and its Application: An Overview. Bulletin of Chemical Reaction Engineering & Catalysis, 7(1), 1-25. https://doi.org/10.9767/bcrec.7.1.1216.1-25

Shahnania M, Mohebbia M, Mehdib A, Ghassempoura A, Aboul-Eneinc HY (2018). Silica microspheres from rice husk: A good opportunity for chromatography stationary phase. Industrial Crops and Products, 121, 236-240. https://doi.org/10.1016/j.indcrop.2018.05.023

Shen Y, Zhao P, Shao Q (2014). Porous silica and carbon derived materials from rice husk pyrolysis char. Microporous and Mesoporous Materials, 188, 46-76. https://doi.org/10.1016/j.micromeso.2014.01.005

Srivastava D, Ali N, Sharma D (2018). Silica Gel, a value added product production from Rice Husk Ash. Journal of Emerging Technologies and Innovative Research, 5(9), 260-266.

Tungkananurak K, Kerdsiri S, Jadsadapattarakul D, Burns DT (2007). Semi-micro preparation and characterization of mesoporous silica microspheres from rice husk sodium silicate using a non-ionic surfactant as a template: application in normal phase HPLC columns. Microchimica Acta, 159(2007), 217-222. https://doi.org/10.1007/s00604-007-0743-x

Xu L, Xia D, Zhang W, Guo Z, Jin G, Zhao Y, Zhang J (2020). Large scale preparation of single chitin oligomers by the combination of homogeneous acid hydrolysis and reversed phase preparative chromatography. Carbohydrate Polymer Technologies and Applications, 1, 100016. https://doi.org/10.1016/j.carpta.2020.100016

Zin YZ (2000). Method for production of silica gel. Patent No. WO2001085614A1 WIPO (PCT). Available at: https://patents.google.com/patent/WO2001085614A1/en

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