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Method of Manufacturing Agar or Agarose Beads

Abstract

The invention discloses method for manufacturing agar or agarose beads, comprising the steps of: a) providing a water phase comprising an aqueous solution of agar or agarose at a temperature of 40-100° C.; b) providing an oil phase comprising a water-immiscible solvent and an emulsifier at a temperature of 40-100° C.; c) emulsifying the water phase in the oil phase to form a water-in-oil emulsion; d) cooling the water-in-oil emulsion to a temperature below a gelation temperature of the agar or agarose to form a dispersion of solidified agar or agarose beads; and e) recovering agar or agarose beads from the dispersion,
wherein the emulsifier comprises a phosphate ester of an alkoxylated fatty alcohol.

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Background/Summary

CROSS REFERENCE TO RELATED APPLICATIONS [0001] This application is a continuation of U.S. application Ser. No. 17/605,502, filed Oct. 21, 2021, which claims the priority benefit of PCT/EP2020/061790, filed on Apr. 28, 2020, which claims the benefit of GB Application No. 1905919.5, filed on Apr. 29, 2019, the entire contents of which are incorporated by reference herein.

TECHNICAL FIELD OF THE INVENTION
[0002] The present invention relates to agar/agarose beads, and more particularly to a method for manufacturing agar or agarose beads. The invention also relates to emulsifiers suitable for use in the method.
BACKGROUND OF THE INVENTION
[0003] Agarose beads have for several decades been used as a stationary phase in chromatographic separations of proteins and other biomacromolecules. They are typically prepared by inverse suspension gelation, where a hot aqueous solution of agarose or agar is emulsified in a hot oil phase to form a water-in-oil (W/O) emulsion. The emulsion is then cooled below the gelation temperature of the agarose/agar to create gel beads, which can then be recovered and used for separation purposes. Such processes are described e.g. in S Hjertén: Biochim Biophys Acta 79 (2), 393-398 (1964), WO1989011493A1 and US20180171484, hereby incorporated by reference in their entireties. A variant where agar beads are converted to agarose beads by hydrolysis of sulfate groups post-gelation is described in US20100084345, also incorporated by reference in its entirety.
[0004] In the process it is essential to use an emulsifier for stabilization of the W/O emulsion. The emulsifier will also be important for the size distribution of the resulting beads and for their shape. Further, the emulsifier should be easy to remove from the beads by washing and it should be environmentally friendly and not give rise to any toxic leachables when the beads are used for manufacturing of pharmaceuticals.
[0005] The emulsifiers previously disclosed are lacking in several of these aspects and accordingly, there is a need for further emulsifiers.
SUMMARY OF THE INVENTION
[0006] One aspect of the invention is to provide a method for the manufacturing agar or agarose beads. This is achieved with a method comprising the steps of: [0007] a) providing a water phase comprising an aqueous solution of agar or agarose at a temperature of 40-100° C.; [0008] b) providing an oil phase comprising a water-immiscible solvent and an emulsifier at a temperature of 40-100° C.; [0009] c) emulsifying the water phase in the oil phase to form a water-in-oil (W/O) emulsion; [0010] d) cooling the W/O emulsion to a temperature below a gelation temperature of the agar or agarose to form a dispersion of solidified agar or agarose beads; and [0011] e) recovering agar or agarose beads from the dispersion, wherein the emulsifier comprises a phosphate ester of an alkoxylated fatty alcohol.
[0012] One advantage is that aggregation of the beads during step d) is prevented, such that well-

dispersed beads of high sphericity are produced. Further advantages are that the emulsifier is water-soluble, facilitating removal by water washing, and that it is free from endocrine disruptors such as alkyl phenol derivatives.
[0013] A further aspect of the invention is to provide agar or agarose beads obtainable by the above method.
[0014] Further suitable embodiments of the invention are described in the dependent claims.

Description

DRAWINGS
[0015] FIG. **1** shows an example of well-dispersed, spherical agarose beads formed from a well-stabilized W/O agarose emulsion.
[0016] FIG. **2** shows an example of agarose beads with aggregates (indicated by arrows) that may form during cooling of a W/O agarose emulsion.
[0017] FIG. **3** shows an example of agarose beads with non-spherical, partially coalesced beads (indicated by arrows).
[0018] FIG. **4** shows an example of agarose beads with spherical inclusions in the beads (indicated by arrows), caused by O/W/O double emulsion formation.
DETAILED DESCRIPTION OF EMBODIMENTS
[0019] In one aspect, the present invention discloses a method for the manufacturing agar or agarose beads. The method comprises the steps of: [0020] a) Providing a water phase comprising an aqueous solution of agar or agarose at a temperature of 40-100° C. The water phase may e.g. comprise 1-8 wt. % agar or agarose, such as 2-7 wt. % agar or agarose, e.g. about 2 wt. %, about 4 wt. % or about 6 wt. %. The water phase can further comprise one or more buffer components such as a weak base. The agar or agarose may be native agar, native agarose or a derivative of agar or agarose, such as e.g. allyl agarose or hydroxyethyl agarose, further described in U.S. Pat. Nos. 6,602,990 and 7,396,467, hereby incorporated by reference in their entireties; [0021] b) Providing an oil phase comprising a water-immiscible solvent and an emulsifier at a temperature of 40-100° C. (suitably at a temperature below the boiling point of the solvent). The water-immiscible solvent can e.g. be a hydrocarbon, an ester or a ketone. To facilitate an efficient solvent recovery, the solvent may have a boiling point in the range of about 90-170° C., such as 90-150° C., or 100-120° C., at atmospheric pressure. It can e.g. be toluene (b.p. 111° C.) or xylene (b.p. about 140° C.: 139° C. for m-xylene and 144° C. for o-xylene). Alternatively, it can be a cyclic ketone, such as 2-methylcyclohexanone (b.p. 162-163° C.). It is also possible to use higher-boiling solvents, such as mineral oils or vegetabilic oils, although the solvent may then be more difficult to recover. The nature of the emulsifier is further described below. The emulsifier may be a single emulsifier or the oil phase may comprise a mixture of several emulsifiers. The concentration of the emulsifier (or the total emulsifier concentration) in the oil phase may e.g. be 0.01-2 wt. %, such as 0.015-1 wt. %. Suitably, none of the emulsifiers comprises alkyl phenols or alkyl phenol derivatives. The oil phase can e.g. comprise less than 0.1 wt %, such as less than 0.01 or less than 0.001 wt % alkyl phenols or alkyl phenol derivatives. It can even be devoid of alkyl phenols and alkyl phenol derivatives; [0022] c) Emulsifying the water phase in the oil phase to form a water-in-oil (W/O) emulsion. The emulsification may comprise mixing the water phase and the oil phase in an agitated vessel to form a W/O emulsion. The step may further comprise passing the W/O emulsion through a rotor-stator mixer, a static mixer or a porous membrane to reduce a droplet size of the W/O emulsion. In an alternative way of forming the emulsion, the water phase can be passed through a porous membrane or sieve plate into the oil phase to form a W/O emulsion; [0023] d) Cooling the W/O emulsion to a temperature below a gelation temperature of the agar or agarose to form a dispersion of solidified agar or agarose beads. This step can be performed by gradually cooling the emulsion

in an agitated vessel or it can be performed in continuous mode by passing the W/O emulsion through a conduit with a longitudinally decreasing temperature gradient; [0024] e) Recovering agar or agarose beads from the dispersion. The recovery may comprise adding water or an aqueous solution to the dispersion obtained in step d), decanting the oil phase and recovering the agar or agarose beads as a sediment. The beads may further be washed with water or an aqueous solution to remove residual emulsifier and/or other substances. Washing with organic solvents can also be applied for removal of emulsifier residues and other leachables.
[0025] After step e), the beads may be crosslinked in a step f), by adding a crosslinking agent, e.g. epichlorohydrin. They may further be functionalized with ligands in a step g), where the ligands are covalently coupled, using methods known to the skilled person. Step g) can suitably be performed after step f), although it is also possible to couple ligands on non-crosslinked beads.
[0026] The beads prepared by the process can be used in chromatographic separation processes or in batch adsorption processes. They may e.g. have diameters (expressed as the volume-weighted median diameter d50,v) in the range of 5-500 μm, such as 10-350 μm or 30-120 μm.
[0027] The emulsifier as mentioned above comprises a phosphate ester of an alkoxylated fatty alcohol. Typically, it can comprise a mixture of phosphate monoester and phosphate diester of the alkoxylated fatty alcohol. The fatty alcohol may comprise one or more C.sub.10-C.sub.20 linear or branched, primary or secondary, alkanols or alkenols and/or the ethoxylated fatty alcohol may have a structure I

R.sub.1—O—(R.sub.2—O).sub.n—H (I) [0028] where: [0029] R.sub.1 is a saturated or unsaturated, linear or branched, aliphatic C.sub.10-C.sub.20 hydrocarbon, such as a saturated or unsaturated linear aliphatic C.sub.10-C.sub.18 hydrocarbon, [0030] R.sub.2 is —CH.sub.2—CH.sub.2— or a mixture of —CH.sub.2—CH.sub.2— and —CH.sub.2(CH.sub.3)—CH.sub.2—, and [0031] n is 2-20, such as 2-10 or 2-5.
[0032] This structure describes an ethoxylated or mixed ethoxylated/propoxylated fatty alcohol, with average alkoxylation degree of n. The alkoxylated fatty alcohol has then been converted into phosphate ester of structure II and III, where II is a phosphate monoester and III is a phosphate diester:

R.sub.1—O—(R.sub.2—O).sub.n—P(O)(OH)—OH (II)

R.sub.1—O—(R.sub.2—O).sub.n—P(O)(OH)—(O—R.sub.2).sub.n—O—R.sub.1 (III) [0033] where: [0034] R.sub.1 is a saturated or unsaturated, linear or branched, aliphatic C.sub.10-C.sub.20 hydrocarbon, such as a saturated or unsaturated linear aliphatic C.sub.10-C.sub.18 hydrocarbon, [0035] R.sub.2 is —CH.sub.2—CH.sub.2— or a mixture of —CH.sub.2—CH.sub.2— and —CH.sub.2 (CH.sub.3)—CH.sub.2—, and [0036] n is 2-20, such as 2-10 or 2-5.
[0037] Both the phosphate monoester II and the diester III are acidic compounds, with acidic hydrogens that can be dissociated. The phosphate ester emulsifiers of the invention can thus be supplied either in acid form or in neutralized form (e.g. as sodium salts or alternatively as potassium or ammonium salts). If emulsifiers in acid form are used, a base may suitably be added to the water phase to adjust the pH to near neutral, as agar and agarose are sensitive to degradation under acidic conditions. In addition to the monoester II and diester III, the emulsifier may also comprise the corresponding phosphate triester of the alkoxylated fatty alcohol and the free, non-esterified alkoxylated fatty alcohol. Both of these compounds are non-acidic and are usually present in minor amounts, such as <25 wt % or less than 15 wt % of the emulsifier.
[0038] In particular, the emulsifier may comprise a mixture of phosphate monoester and phosphate diester of a mixed ethylene oxide+propylene oxide adduct of a C.sub.10-C.sub.16 alkanol. Such a product is commercially available under the name of Lubrhophos™ LF-800 (Solvay). Alternatively, the emulsifier may comprise a mixture of phosphate monoester and phosphate diester

of ethoxylated oleyl alcohol with n=3 (average number of ethylene glycol units per oleyl alcohol). Such a product is generally known under the INCI (International Nomenclature of Cosmetic Ingredients) name of oleth-3 phosphate and is commercially available under the trade name of Crodafos™ 03A (Croda).

EXAMPLES

Emulsification Method

[0039] A solution of 35 g agarose in 490 ml of water was prepared at 95° C. and subsequently cooled to 70° C. after addition of 7.0 mM phosphate to give pH 7.0. A solution of emulsifier in 850 ml toluene was prepared and heated to 60° C. in a 3 L thermostated jacketed cylindrical glass reactor. Under agitation with an overhead agitator, the agarose solution was added to the reactor under 80 rpm agitation and the agitation was continued with stepwise increasing rpm at 60° C. until the agarose droplet size was approximately 100 μm, as assessed from samples removed and analysed by laser diffraction. These samples were rapidly cooled with ice to avoid any coalescence/aggregation before the analysis. The temperature of the reactor jacket was then lowered to 20° C. to solidify the agarose droplets. The resulting agarose beads were washed with toluene and/or water, the washing liquids were decanted while the agarose beads were recovered as a sediment.

Evaluation Methods

[0040] The particle size distribution was measured using a Mastersizer 3000 laser diffraction instrument (Malvern Panalytical) for agarose beads in an ethanol dispersion with ethyl cellulose as a dispersant. The distributions were plotted as differential volume vs diameter curves and the mode of each distribution was calculated by the instrument. The mode is the peak of the distribution, i.e. the highest peak seen in the distribution curve. The mode thus represents the particle size most commonly found in the distribution. Samples were taken both before and after the cooling of the emulsions and the difference between the mode after cooling and the mode before cooling was denoted A mode. This is a measure of the particle size increase during cooling, indicative of coalescence and/or aggregation occurring during the sensitive cooling phase.

[0041] The beads were also evaluated visually in a microscope with respect to inclusions in the beads (which may result from oil-in-water-in-oil double emulsion formation) and spherical shape, where deviations from spherical shape may be due to partial coalescence of droplets. Examples of aggregates, non-spherical beads and beads with inclusions are shown in FIGS. **2**-**4**.

Emulsifiers

TABLE-US-00001 TABLE 1 Emulsifiers used. Fatty Ethylene Product alcohol oxide name Supplier Chemical structure moiety units (n) Lubrhophos Solvay C10-16 ethoxylated/ C10-16 LF-800 propoxylated phosphate Lubrhophos Solvay Polyoxyethylene oleyl C16-18:1 5 LB-400 ether phosphate Rhodafac Solvay Polyoxyethylene oleyl C16-18:1 2 PA/32 ether phosphate Rhodafac Solvay Polyoxyethylene oleyl C16-18:1 5 PA/35 ether phosphate Crodafos Croda PPG-5-ceteth-10 C16 10(PO5) SG-LQ phosphate Crodafos Croda Oleth-10 phosphate C18:1 10 O10A Crodafos Croda Oleth-3 phosphate C18:1 3 O3A Crodafos Croda Ceteareth-2 phosphate C16-18 2 CS2A Lakeland Lakeland Phosphate ester of C18 5 PAE 185 ethoxylated octadecanol 5 EO Hostaphat Clariant Lauryl polyethoxy C12 4 KL340D (4EO) phosphate Rhodafac Solvay Phosphate ester of 10 RM-510 dinonylphenol ethoxylate SPAN 60 Croda Sorbitan monostearate SPAN 80 Croda Sorbitan monooleate SPAN 120 Croda Sorbitan isostearate

Example 1—Emulsifier Comparisons

TABLE-US-00002 TABLE 2 Emulsification results Conc. emulsifier Max in toluene stirring Mode prior Δ phase Inclusions Spherical Emulsification speed to cooling mode Emulsifier (wt./vol %) (Y/N) (Y/N) time (min) (rpm) (μm) (μm) Lubrhophos LF- 0.016 N Y 1450 234 57 800 Lubrhophos LF- 0.05 N Y 157 1350 103 35 800 Lubrhophos LF- 0.08 N Y 105 1450 96 8 800 Lubrhophos LF- 0.16 N Y 800 19 17 800 Lubrhophos LF- 0.20 N Y 800 15 6 800 Lubrhophos LB- 0.016 Y N 106 1500 177 143 400 Lubrhophos LB- 0.05 N Y 168 1350 106 86 400 Lubrhophos LB- 0.16 N Y 100

1450 97 17 400 Rhodafac PA/32 0.16 Y Y 151 1600 108 20 Rhodafac PA/35 0.16 N Y 108 1550 108 12 Crodafos SG- 0.16 N N 144 1600 115 98 LQ Crodafos SG- 0.16 N N 118 1550 103 45 LQ Crodafos O10A 0.16 Y N 148 1650 161 548 Crodafos O3A 0.16 N Y 87 1300 102 5 Crodafos CS2A 0.16 N Y 70 1250 21 2 Lakeland PAE 0.16 Y N 153 1650 125 130 185 Hostaphat 0.16 N Y 64 1200 112 34 KL340D Rhodafac RM- 0.16 N Y 74 1350 96 25 510 Span 60 0.2 Y N 218 1550 778 176 Span 80 0.1 Y N 156 1250 559 Span 80 0.2 Y N 148 1200 810 Span 80 0.5 Y N 195 1600 218 123 Span 80 2 N Y 70 1250 99 5 Span 120 0.2 Y N 139 1250 907 Span 120 0.5 N Y 118 1450 413 −102 Span 120 2.3 N Y 111 1450 100 7

[0042] Basically, all the tested materials function as emulsifiers. There are however differences between them. The nonionic emulsifiers, as exemplified by the three sorbitan esters, have to be used at concentrations of 2% to give good results. This means that a high amount of emulsifier has to be washed out from the beads during the recovery, which is undesirable, in particular since those emulsifiers are not water soluble and have to be washed out with solvents. Phosphate esters can be used at lower concentrations (below 0.2%) and are generally water soluble. Particularly good results were obtained with Lubrhophos LF-800, Lubrhophos LB-400, Rhodafac PA/35, Crodafos CS2A and Crodafos 03A, with Lubrhophos LF-800 and Crodafos O3A selected as top candidates. Rhodafac RM-510 also gave good results but was deselected for environmental reasons, as it is based on alkylphenol ethoxylates.

Example 2—2-methylcyclohexanone as Solvent

[0043] Two experiments were carried out as above, but with 2-methylcyclohexanone instead of toluene. The emulsifier was Lubrhophos LF-800 and the emulsifier amounts were 0.017 wt./vol. % and 0.23 wt./vol. % respectively. The target particle size of 100 μm was reached and the visual appearance of the beads was good. Some aggregation occurred during cooling though.

[0044] This written description uses examples to disclose the invention, including the best mode, and also to enable any person skilled in the art to practice the invention, including making and using any devices or systems and performing any incorporated methods. The patentable scope of the invention is defined by the claims, and may include other examples that occur to those skilled in the art. Such other examples are intended to be within the scope of the claims if they have structural elements that do not differ from the literal language of the claims, or if they include equivalent structural elements with insubstantial differences from the literal languages of the claims. All patents and patent applications mentioned in the text are hereby incorporated by reference in their entireties as if individually incorporated.

Claims

1-**28**. (canceled)

29. A method for environmentally friendly manufacturing of agar or agarose beads, the method comprising emulsifying a water phase comprising an aqueous solution of agar or agarose in an oil phase comprising a water-immiscible solvent and an emulsifier, wherein the emulsifier comprises a phosphate ester of an alkoxylated fatty alcohol.

30. The method of claim 29, wherein said oil phase comprises less than 0.1 wt. % of alkyl phenols or alkyl phenol derivatives.

31. The method of claim 29, wherein said oil phase is devoid of, or essentially devoid of, alkyl phenols and alkyl phenol derivatives.

32. The method of claim 29, wherein said emulsifier comprises a mixture of phosphate monoester and phosphate diester of said alkoxylated fatty alcohol.

33. The method of claim 29, wherein said fatty alcohol comprises one or more C.sub.10-C.sub.20 linear or branched, primary or secondary, alkanols or alkenols.

34. The method of claim 29, wherein said alkoxylated fatty alcohol has a structure R.sub.1—O—(R.sub.2—O).sub.n—H (I) wherein: R.sub.1 is a saturated or unsaturated, linear

or branched, aliphatic C.sub.10-C.sub.20 hydrocarbon, R.sub.2 is —CH.sub.2—CH.sub.2— or a mixture of —CH.sub.2—CH.sub.2— and —CH.sub.2 (CH.sub.3)—CH.sub.2—, and n is 2-20.

35. The method of claim 29, wherein said emulsifier comprises a mixture of R.sub.1—O—(R.sub.2—O).sub.n—P(O)(OH)—OH and (II) R.sub.1—O—(R.sub.2—O).sub.n—P(O)(OH)—(O—R.sub.2).sub.n—O—R.sub.1 (III) wherein: R.sub.1 is a saturated or unsaturated, linear or branched, aliphatic C.sub.10-C.sub.20 hydrocarbon, R.sub.2 is —CH.sub.2—CH.sub.2— or a mixture of —CH.sub.2—CH.sub.2— and —CH.sub.2 (CH.sub.3)—CH.sub.2—, and n is 2-20.

36. The method of claim 34, wherein R.sub.1 is a saturated or unsaturated linear aliphatic C.sub.10-C.sub.18 hydrocarbon.

37. The method of claim 29, wherein the water phase and the oil phase are emulsified to a water-in-oil emulsion at a temperature of 40-100° C.

38. The method of claim 37, wherein the water-in-oil emulsion is cooled to a temperature below a gelation temperature of said agar or agarose to form a dispersion of solidified agar or agarose beads that may be recovered from said dispersion.

39. The method of claim 29, wherein the emulsifying comprises mixing said water phase and said oil phase in an agitated vessel to form a water-in-oil emulsion.

40. The method of claim 39, further comprising passing said water-in-oil emulsion through a rotor-stator mixer to reduce a droplet size of said water-in-oil emulsion.

41. The method of claim 39, further comprising passing said water-in-oil emulsion through a static mixer to reduce a droplet size of said water-in-oil emulsion.

42. The method of claim 39, further comprising passing said water-in-oil emulsion through a porous membrane to reduce a droplet size of said water-in-oil emulsion.

43. The method of claim 29, wherein the agar or agarose beads are recovered by washing said agar or agarose beads with water or an aqueous solution to remove residual emulsifier.

44. Environmentally friendly, well-dispersed and highly spherical agar or agarose beads, obtainable by the method of claim 29.