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Iron oxide superparamagnetic nanocarriers bearing amphiphilic N-heterocyclic choline analogues as potential antimicrobial agents†

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Magnetic nanoparticles represent an advanced tool in biomedicine because they can be simultaneously functionalized and guided using a magnetic field. Iron oxide magnetic nanoparticles precoated with oleic acid and bearing novel antimicrobial N-heterocyclic choline analogues, namely *O*-, *N*- and *O*,*N*-bis-undecyl-substituted *N*-(2-hydroxyethyl)-1,2,3,4-tetrahydroisoquinolinium derivatives, have been obtained as potential biomedical agents for drug delivery and antimicrobial therapy. Structural and size determinations for the novel synthesized magnetic nanosystems were carried out based upon magnetogranulometry, dynamic light-scattering measurements and X-ray diffraction analysis. The most expected iron oxide core diameter was 6.2–10.5 nm. The magnetization analyses showed that the particles are superparamagnetic at room temperature. Aqueous magnetic fluids of the synthesized nanoparticles were examined *in vitro* concerning Gram-positive (*Staphylococcus aureus* MSCL 334, *Bacillus cereus* MSCL 330) and Gram-negative (*Escherichia coli* MSCL 332, *Pseudomonas aeruginosa* MSCL 331, *Proteus mirabilis* MSCL 590) bacterial strains and fungi (*Candida albicans* MSCL 378, *Aspergillus niger* MSCL 324). It was found that the samples have magnetic properties and possess antimicrobial activity. The minimum inhibitory concentration against *S. aureus* for the most active magnetic fluid was determined as 16 μg ml^{-1}.

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Keywords: magnetic nanoparticles; magnetic fluids; iron oxide; 1,2,3,4-tetrahydroisoquinoline; antimicrobial activity

Introduction

A major problem in drug therapy is the lack of specificity of therapeutic agents towards the site of disease localization (drug delivery). In recent years engineered magnetic nanoparticles (NPs) have come to represent an advanced tool in biomedicine because they can be simultaneously functionalized and guided using a magnetic field. Progress in nanometre-sized magnetic particles has opened new opportunities for their medical applications, including drug delivery, diagnostics, hyperthermia therapy, immunology, molecular biology, DNA purification, cell separation and purification.[1–3] Most of these promising applications require well-defined and controllable interactions between the magnetic NPs and living cells. Also, the biological action of metal oxide NPs is size-dependent.[4]

Our efforts are focused on searching for medical remedies based on iron oxide/oleic acid nanocarriers with immobilized small molecules,[5] possessing various kinds of biological activity and on developing strategies for targeted drug delivery including both molecular (suitable coating) and magnetic (iron oxide core) targeting systems. Considering coating materials for drug delivery applications, it is usually required that particles have sufficiently hydrophilic surfaces, relatively small sizes, so they can evade the reticulo-endothelial system, and to be nontoxic.[6,7] The original design is based on principles similar to the structure of natural magnetosomes,[8] where the first biologically active substance is covalently coupled to the magnetic core and the second amphiphilic biologically active ligand is subsequently immobilized on the surface of the magnetic carrier by creating plasma membrane-like structure around the magnetic core (Fig. 1). It can be speculated that thus created systems are able to provide an interaction with cells via particle shell and cell membrane fusion. Also, these complex compositions can be accepted as typical pro-drugs due to the structure, which is capable of ensuring a prolonged action by the process of gradual desorption, not destroying the whole molecule immediately. As the result of a selective approach, which includes targeted modification for definite type of activity, we intend to obtain new potential theranostics[9] for therapeutic (hypothermia, weak magnetic fields, direct therapeutic effect of the ligand) and/or diagnostic (magnetic resonance imaging) applications. In our previous research the procedure for the synthesis of model magnetic NPs with immobilized cytotoxic organosilicon

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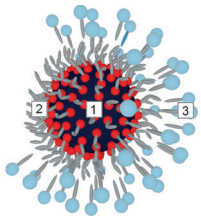


(1) – $FeO{\cdot}Fe_2O_3$; (2) – $CH_3(CH_2)_7CH{=}CH(CH_2)_7COOH$; (3) – $[\mathbf{ROCH_2CH_2}NR'R''R'''R'''']^+$

Figure 1. Schematic illustration of designed nanoparticles.

choline and colamine derivatives, accepted as silyl pro-drugs,[10,11] has been developed and their water-based magnetic fluids (MFs) have been prepared.[12] It has been demonstrated that the resulting MFs had magnetic properties and affected tumour cell lines.[13,14] The positive results obtained stimulated us to extend the research to more complex biologically active structures and to other types of biological activity.

The aim of the investigation reported here was the design, synthesis and determination of morphology and biological study of mixed covered magnetic nanostructures assembling a final product composed of natural components: magnetite (Fe_3O_4) as a magnetic core; oleic acid (OA) as biocompatible shell; and, anchored to the surface, newly synthesized molecules of lipid-like choline analogues possessing antimicrobial properties. Interest in amphiphilic compounds, which we used as ligands for the preparation of NPs, is based on the fact that structural restrictions, caused by long-chain alkyl substituents and positively charged quaternized nitrogen atoms, may influence the coordination behaviour of these compounds in their possible reaction with biological nucleophiles and complex molecules, and may affect the adsorption process on cell surfaces.[15] Quaternary ammonium salts are frequently used as antibacterial agents that disrupt the cell membrane through the binding of their ammonium cations to anionic sites in the outer layer tissue of bacteria. It has been shown that the antimicrobial activity of some tetraalkylammonium salts is under the control of their molecular hydrophobicity, adsorbability, surface activity and electron density of the ammonium nitrogen atom.[16] The use of the choline moiety in the design of molecules for NP shells is justified by its biological function in the structural integrity of cell membranes and its identification as an allosteric ligand necessary for recognition and degradation of cell walls by pneumococcal amidase.[17] In addition, we have found that all the classes of natural and synthetic antimicrobial agents contain characteristic choline or colamine –OCCN= atom sequence.[18] Synthesis of amphiphilic ligands was carried out by introduction of lipophilic substituents via *O,N*-alkylation of a heterocyclic choline analogue, where trimethylammonium group is replaced with tetrahydroisoquinoline ring system, which is an important structural motif and is commonly encountered in naturally occurring anti-infective alkaloids.[19,20] Also, 1,2,3,4-tetrahydroisoquinoline-based fragments are accepted as versatile scaffolds of the lead compounds for the development of plasma membrane transporter P-gp modulating molecules, connected with multi-drug resistance.[21]

Experimental

Chemicals and Instrumentation

^{1}H NMR and ^{13}C NMR spectra were obtained with Varian Mercury 200 and Varian Mercury 400 spectrometers with $CDCl_3$ as a solvent and with HMDSO ($\delta = 0.055$ ppm) as internal standard. Mass spectra under electron impact conditions were recorded with an Agilent Technologies mass spectrometer 5975C (GC 7890A, 70 eV) and a Waters mass spectrometer 3100 (LC Alliance Waters 2695). Elemental analyses (C, H, N) were performed with a Carlo Erba 1108 elemental analyser. Elemental analysis results agreed with calculated values. Melting points were determined using a Boetius melting point apparatus and were taken uncorrected. Analytical thin-layer chromatography (TLC) was performed on 60 F_{254} (Merck) silica gel plates. Column chromatography was performed using Merck silica gel (0.040–0.063 nm). Solvents and reagents used in this study were purchased from Fluka, Acros and Sigma-Aldrich. All solvents used were freshly dried using standard techniques and all glassware was oven-dried. The dependence of magnetization on external magnetic field for the samples was determined using a vibrating sample magnetometer (Lake Shore Cryotronics, Inc., model 7404). X-ray diffraction patterns were measured with an X'PertPro MPD (PANalytical BV) diffractometer with PIXcel linear detector. The size of particles with solvate shell was measured using dynamic light scattering (DLS; ZEN1690, Malvern Instruments Ltd).

Synthesis of Ligands

N-(2-Hydroxyethyl)-1,2,3,4-tetrahydroisoquinoline (**1**)[22] was characterized by comparing its ^{1}H NMR spectrum with those reported in the literature.

*N-(2-n-Undecyloxyethyl)-1,2,3,4-tetrahydroisoquinoline (**2**)*

A mixture of **1** (0.5 g, 2.8 mmol), 1-chloroundecane (0.53 g, 2.8 mmol) and sodium metal (0.06 g, 2.8 mmol) in 1 ml of dry benzene was heated at 78 °C with stirring for 2 h under argon. The course of the reaction was followed using GC-MS. Once the reaction had reached completion, the reaction mixture was cooled to room temperature and the solid traces were filtered off. The resulting crude residue was purified using column chromatography on silica gel eluted with chloroform–methanol (10:1) to give the desired undecyl ether as a dark yellow oily liquid.

Yield 0.54 g (60%). GC-MS (*m/z*, %): 331 (M^+, 2), 160 ($M^+ - OC_{11}H_{23}$, 4), 146 ($M^+ - CH_2OC_{11}H_{23}$, 100). ^{1}H NMR ($CDCl_3$, δ, ppm): 0.87 (3H, t, $J = 6.8$ Hz, CH_3), 1.2–1.3 (16H, bs, CH_2), 1.5–1.6 (2H, m, CH_2), 2.75 (2H, t, $J = 6.0$ Hz, α-CH_2N), 2.80 (2H, t, $J = 5.8$ Hz, 4-CH_2), 2.89 (2H, t, $J = 5.8$ Hz, 3-CH_2), 3.44 (2H, t, $J = 6.6$ Hz, OCH_2), 3.64 (2H, t, $J = 6.0$ Hz, β-CH_2O), 3.69 (2H, s, 1-CH_2N), 7.0 (1H, m, 8-H), 7.2 (3H, m, 5,6,7-H). ^{13}C NMR ($CDCl_3$, δ, ppm): 14.10 (CH_3); 22.67, 26.17, 28.99, 29.32, 29.48, 29.61, 29.65 (CH_2); 31.90 (4-CH_2); 51.41(3-CH_2N); 56.50 (1-CH_2N); 57.59 (α-CH_2N); 69.13, 71.78 (OCH_2); 125.51 (6-C), 126.02 (7-C), 126.54 (8-C), 128.60 (5-C); 134.20 (9-C), 134.83 (10-C). Anal. Calcd for $C_{22}H_{37}NO$ (%): C, 79.70; H, 11.25; N, 4.22. Found (%): C, 79.85; H, 11.21; N, 4.25.

*N-Methyl-N-(2-n-undecyloxyethyl)-1,2,3,4-tetrahydroisoquinolinium iodide (**3**)*

A solution of undecyl ether **4** (0.35 g, 1.1 mmol) in dry diethyl ether (5 ml) was heated at 35 °C for 4 h with methyl iodide (0.47 g, 3.3 mmol). After cooling to room temperature the reaction mixture was filtered, the solid was washed with diethyl ether and dried for 6 h in vacuum to give the desired methiodide as a yellow powder.

Yield 0.26 g (50%); m.p. 52–54 °C. GC-MS (*m/z*, %): 331 ($M^+ - MeI$, 1), 146 ($M^+ - MeI - CH_2OC_{11}H_{23}$, 100). ^{1}H NMR ($CDCl_3$, δ, ppm): 0.87 (3H, t, $J = 6.8$ Hz, CH_3), 1.25 (16H, bs, CH_2), 1.56 (2H, m, CH_2), 3.25 (2H, m, 4-CH_2), 3.48 (2H, t, $J = 6.4$ Hz, OCH_2), 3.54 (3H, s, N^+CH_3), 3.95 (2H, m, α-CH_2N^+), 4.05 (2H, m, β-OCH_2), 4.1–4.2 (2H, m, 3-CH_2N^+), 4.83 and 5.05 (total 2H, d and d, $J = 15.0$ Hz, 1-CH_2N^+), 7.14 (1H, d, $J = 7.3$, Hz, 8-H), 7.29 (3H, m, 5,6,7-H). ^{13}C NMR ($CDCl_3$, δ, ppm): 14.03

(CH_3); 23.90 (4-CH_2); 22.64, 26.13, 29.29, 29.35, 29.41, 29.56, 31.86 (CH_2); 48.97 (N^+CH_3); 59.64, 61.78, 63.70, 64.49 (1,3,α-CH_2N^+ and β-CH_2O); 71.99 (OCH_2); 125.94 (9-C), 127.41 (8-C), 127.82 (5-C), 128.56 (6-C); 128.90 (7-C), 129.08 (10-C). Anal. Calcd for $C_{23}H_{40}INO$ (%): C, 58.34; H, 8.52; N, 2.96. Found (%): C, 58.27; H, 8.48; N, 2.98.

*N-(2-Hydroxyethyl)-N-n-undecyl-1,2,3,4-tetrahydroisoquinolinium iodide (**4**)*

A mixture of **1** (0.2 g, 1.1 mmol) and 1-iodoundecane (0.71 g, 2.2 mmol) in 3 ml of dry MeCN was heated at 81 °C under stirring for 8 h. The course of the reaction was followed using GC-MS. Once the reaction had reached completion, the reaction mixture was cooled to room temperature. The precipitate was filtered off, washed with diethyl ether and dried in vacuum to give the product as a white powder.

Yield 0.32 g (63%); m.p. 110–112 °C. GC-MS (*m/z*, %): 459 (M^+), 334 ($M^+ - I + 1$, 1%), 282 ($M^+ - I - CH_2OH - 1$, 6), 146 ($M^+ - I - C_{11}H_{23} - CH_2OH + 1$, 100). LC-MS (*m/z*, %): 333 ($M^+ - I$, 100). ^{1}H NMR ($CDCl_3$, δ, ppm): 0.87 (3H, t, J = 6.8 Hz, CH_3), 1.23 (16H, bs, CH_2), 1.84 (2H, m, CH_2), 3.18 (2H, m, 4-CH_2), 3.64, 3.81, 3.90 and 4.18 (total 8H, all m, α,α′-CH_2N^+, 3-CH_2N^+ and β-CH_2O), 4.78 and 4.92 (total 2H, d and d, J = 15.2 Hz, 1-CH_2N^+), 7.2–7.4 (4H, m, 5,6,7,8-H). ^{13}C NMR ($CDCl_3$, δ, ppm): 14.09 (CH_3); 23.83 (4-CH_2); 22.53, 22.65; 29.14, 29.26, 29.37, 29.42, 29.52, 29.60, 29.84 (CH_2); 55.39 (α′-CH_2N^+); 57.19, 59.62, 61.06, 62.13 (1,3,α-CH_2N^+ and OCH_2); 125.85, 127.56, 127.91, 128.84 (5,6,8,9-C); 128.92 (7-C), 129.07 (10-C). Anal. Calcd for $C_{22}H_{38}INO$ (%): C, 57.51; H, 8.34; N, 3.05. Found (%): C, 57.44; H, 8.33; N, 3.03.

*N-(2-n-Undecyloxyethyl)-N-n-undecyl-1,2,3,4-tetrahydroisoquinolinium iodide (**5**)*

A solution of undecyl ether **2** (0.20 g, 0.6 mmol) in dry MeCN (3 ml) was heated at 80 °C for 8 h with 1-iodoundecane (0.34 g, 1.2 mmol). After cooling to room temperature the reaction mixture was filtered, the solid was washed with ether and dried in vacuum for 4 h to give the desired product as a light yellow powder.

Yield 0.09 g (24%); m.p. 144–146 °C. GC-MS (*m/z*, %): 456 ($M^+ - MeI - Me - 1$, 1), 146 ($M^+ - C_{11}H_{23} - CH_2OC_{11}H_{23}$, 100). ^{1}H NMR ($CDCl_3$, δ, ppm): 0.87 (6H, t, J = 6.7 Hz,CH_3), 1.25 (32H, bs, CH_2), 1.55 (4H, m, CH_2), 3.18 (2H, m, 4-CH_2), 3.48 (2H, t, J = 6.5 Hz, OCH_2), 3.55 and 3.94 (6H, all m, α,α′-CH_2N^+ and β-CH_2O), 4.19 (2H, m, 3-CH_3N^+), 4.68 and 5.14 (total 2H, d and d, J = 15.4 Hz, 1-CH_2N^+), 7.15 (1H, d, J = 7.4 Hz, 8-H), 7.2–7.4 (3H, m, 5,6,7-H). ^{13}C NMR ($CDCl_3$, δ, ppm): 14.07 (CH_3); 22.64, 23.78, 26.18, 29.14–29.59 (CH_2), 31.84 (4-CH_2), 56.80, 59.29, 59.57, 60.54, 61.50 (α,α′,1,3-CH_2N^+ and OCH_2), 72.05 (CH_2, OCH_2); 125.85, 127.51, 127.92, 128.83, 129.09 (5,6,7,8,9,10-C). Anal. Calcd for $C_{33}H_{60}INO$ (%): C, 64.58; H, 9.85; N, 2.28. Found (%): C, 64.67; H, 9.81; N, 2.27.

Synthesis of Nanoparticles

Magnetite (Fe_3O_4) NPs **S1** were obtained using wet synthesis by precipitation from an aqueous solution of Fe(II) sulfate and Fe(III) chloride with excess sodium hydroxide. MFs **S2** (**S2a–S2d**) were obtained by reaction of the prepared magnetite NPs **S1** with the first surfactant, namely OA in toluene,[12,23] and differed in their content. MF **S2c** was prepared from the residue left after MF **S2b** decantation by sonication of its toluene solution. Powders **S3** (**S3a–S3d**) were prepared from the corresponding MFs **S2** (**S2a–d**) by treatment with acetone after toluene evaporation. Magnetic powders **S8–S11** were prepared by shaking MFs **S2** with corresponding tetrahydroisoquinoline ligands **3–5** or additional quantity of OA, and consecutive treating of obtained MFs **S4–S7** with acetone after toluene evaporation. Magnetic powders **S8–S11** were treated with water to produce water solutions **S12–S15**. Water-soluble powders **S16–S19** were obtained by solvent evaporation from the corresponding water solutions. The yield of water-soluble NPs **S16–S19** was calculated relative to the initial amount of powders **S8–S11**. The magnitude of magnetization and magnetite concentration were calculated based on the same parameters obtained for the corresponding water solutions. MF **S2a** was the starting material for preparation of the series of samples **S4a–S17a**; **S2b** for samples **S4b–S19b**; and **S2c** for samples **S4c–S16c**.

X-Ray Analysis

The structure of initial synthesized magnetite (Fe_3O_4) NPs was determined using X-ray diffraction analysis with the database ICDD PDF-4 and HighScore software. XRD patterns were measured in flat Bragg–Brentano geometry; Soller slits, 0.04 rad; filtered Cu Kα radiation; generator settings, 30 mA, 40 kV; continuous scan type; 2θ interval, 10–90°; step size, 0.026 2θ; scan step time, 50 s.

Magnetic Measurements

Magnetogranulometry was used for investigation of magnetic properties and determination of the diameter of the iron oxide magnetic core of the prepared NPs. Magnetic properties of MFs were studied in solutions without separation of carrier fluid. A special glass container with MF was placed in the measuring system of the magnetometer. Magnetization curves were recorded at different stages of material treatment for monitoring of magnetite concentration and its condition as well. As a rule, measurements were done at room temperature for fields to 10 kOe. The magnetization curves were used for the magnetogranulometry analysis[24] to find magnetization of superparamagnetic saturation and magnetic moment of magnetite particles in a sample from which the magnetite concentration and the particle sizes in the sample were calculated. Magnetogranulometry gives the particle size distribution. The most expected particle size was used as a particle diameter (d). For distribution width estimation, d_{min} and d_{max} at level 0.5 from distribution were used. To analyse the weak magnetic samples, the matrix (water or toluene) at the same holder was measured separately. The matrix magnetization was subtracted from the full magnetization.

Dynamic Light Scattering Measurements

DLS, sometimes referred to as photon correlation spectroscopy or quasi-elastic light scattering, is a technique for measuring the size of particles typically in the sub-micrometre region. In DLS the speed at which particles diffuse due to Brownian motion is measured using a laser. DLS gives the colloidal particle size distribution based on translational diffusion coefficients in the carrier liquid (the particles are modelled as spheres). Measurements were done at 20 °C.

Biological Tests

Agar diffusion test

For the determination of antimicrobial activity, several reference microbial strains, received from the Microbial Strain Collection of Latvia (MSCL), Riga, Latvia, were used. Bacteria: *Staphylococcus aureus* MSCL 334 (SA), *Bacillus cereus* MSCL 330 (BC), *Proteus mirabilis* MSCL 590 (PM), *Escherichia coli* MSCL 332 (EC) and *Pseudomonas aeruginosa* MSCL 331 (PA); fungi: *Candida albicans* MSCL 378 (CA)

and *Aspergillus niger* MSCL 324 (AN). All bacteria were cultivated on plate count agar (Sanofi Diagnostics Pasteur, France) at 37 °C for 24 h. CA was cultivated on Difco™ malt extract agar (Becton, Dickinson and Company, UK) at 37 °C for 48 h. Antimicrobial activity was determined using the agar well diffusion method.[25] The agar diffusion test was performed on Müller–Hinton (Carl Roth GmbH Co. KG, Germany) agar for bacteria and malt extract agar for yeasts. Suspensions of 18–24 h microbial cultures of turbidity A_{540} = 0.16 ± 0.20 were used and uniformly spread on Petri plates. Aliquots of 70 µl of each test sample solution in dimethylsulfoxide, corresponding solvent and reference antimicrobial drug solutions were added to 6.0 µm diameter agar wells. Gentamicin (KRKA, Slovenia) and fluconazole (Diflucan, Pfizer Ltd, UK), 10 and 5 mg ml^{-1}, were used as reference antibiotics. The antimicrobial activity was taken on the basis of the diameter of zone of inhibition. After incubation at 37 °C for 24 h for bacteria and 48 h for yeasts under aerobic conditions, the diameter of the clear zone (no growth) around the well in the bacterial lawn was measured. The inhibition zone diameter was measured in millimetres. The tests were performed in duplicate. The results were expressed as the arithmetic average.

Broth dilution assay

For the determination of antimicrobial activity, *Staphylococcus aureus* MSCL 334 was used as reference microbial strain, received from the Microbial Strain Collection of Latvia (MSCL, Riga, Latvia). Bacteria were cultivated on plate count agar medium (Sanofi Diagnostics Pasteur, France) at 37 °C for 24 h. Antimicrobial activity was determined using the broth dilution method. Minimal inhibitory concentration (MIC) against the bacterial strain was determined by a standardized dilution method using Müller–Hinton broth (BD Difco™, UK). MIC was determined using medium RPMI-1640 with L-glutamine but without bicarbonate (Sigma-Aldrich, UK). In order to obtain a final density of approximately 10^6 colony-forming units per millilitre, test strains were suspended in sterile water. Series of dilution tubes were prepared and incubated at 37 °C for 24 h. The MIC was defined as the lowest concentration of antimicrobial agent that inhibited the growth of the microorganisms detected by the lack of visual turbidity, matching with a negative control included in the test. Following a broth dilution MIC test, from each tube that showed no growth, 50 µl of suspension was removed and spread onto appropriate agar plates and incubated at 37 °C for 24 h.[26]

Results and Discussion

The present work describes the synthesis and physicochemical and antimicrobial properties of new magnetite-based NPs with the general formula displayed in Scheme 1, covered with biologically active ligands: OA and **3**, **4** or **5**.

Preparation of aqueous magnetic colloids of iron oxide NPs for biological investigation includes: (a) synthesis of new amphiphilic N-heterocyclic choline derivatives and investigation of their antimicrobial properties and (b) synthesis and physicochemical investigation of iron oxide magnetic NPs, coated with biologically active substances, including immobilization of the modified choline derivatives on the surface of the magnetic nanocarriers; preparation of their colloidal solutions in organic solvent; obtaining powders for physicochemical investigations; and preparation of water solutions to study physicochemical and biological properties.

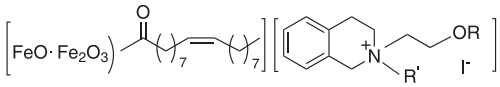
Synthesis of new biologically active tetrahydroisoquinolinium compounds **3–5** (Scheme 2) was carried out by chemical modification of **1** via introduction of long-chain alkyl substituents into the molecule.

Intermediate **1** was synthesized from the commercially available materials 1,2,3,4-tetrahydroisoquinoline and ethylene bromohydrin according to a method previously described[22] and was modified by introduction of undecyl substituent by alkylation of either side chain or heterocyclic nitrogen atom with 1-iodoundecane to provide the products of *O*-alkylation (**3**) and *N*-alkylation (**4**). Ether **2** was also converted to *O*,*N*-bis-undecyl-substituted tetrahydroisoquinolinium derivative **5**.

Antimicrobial activity of the synthesized ligands **3–5** was investigated against two Gram-positive bacteria (SA and BC), three Gram-negative bacteria (EC, PA and PM) and two fungi strains (CA and AN), using the agar dish diffusion method[25,27] and was examined quantitatively by measuring of the diameter of the inhibition zone in comparison with gentamicin and fluconazole as reference compounds.

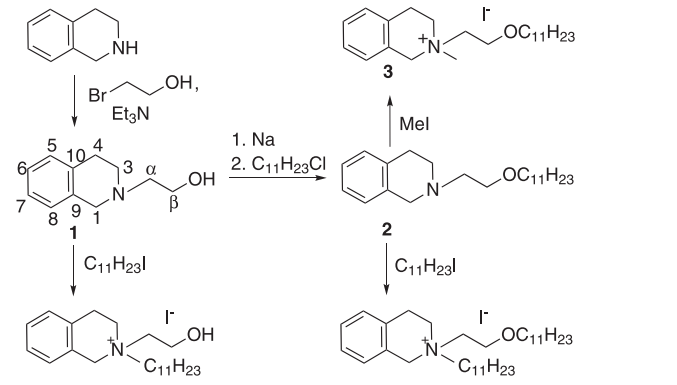
The investigation of antimicrobial screening (Table 1) reveals that the newly synthesized compounds **3** and **4** show strong and highly selective antibacterial activity against Gram-positive SA and BC, with diameters of inhibition zones ranging from 22 to 33 mm, and moderate antibacterial activity with inhibition zones ranging from 15 to 20 mm against Gram-negative EC, PA and PM. The compounds are also potent against fungal strains CA and AN exhibiting diameters of inhibition zones from 17 to 24 mm and from 16 to 21 mm, respectively. Compound **5** shows weak antimicrobial activity against Gram-positive SA and BC and fungus CA and is almost inactive against Gram-negative bacteria. Compound **5** is most potent against fungal strain AN with an inhibition zone diameter of 15 mm. OA lacked antimicrobial activity against all microorganisms under investigation.

The screened amphiphilic heterocyclic choline analogues **3–5** were further used as ligands (second surfactant) in the synthesis



R = H, $C_{11}H_{23}$; R' = CH_3, $C_{11}H_{23}$

Scheme 1. General formula of mixed covered iron oxide nanoparticles.



Scheme 2. Synthesis of ligands **3–5** and iron oxide/oleic acid-based nanoparticles.

Table 1. *In vitro* antibacterial and antifungal activity data of *N*-(2-hydroxyethyl)-1,2,3,4-tetrahydroisoquinoline derivatives **3–5** and OA

Compound	Diameter of zones showing complete inhibition of growth (mm)[a]													
	SA		BC		EC		PA		PM		CA		AN	
	Concentration of compound (mg ml^{-1})													
	5	10	5	10	5	10	5	10	5	10	5	10	5	10
OA	–	–	–	–	–	–	–	–	–	–	–	–	–	–
3	23	25	22	22	20	20	12	14	15	15	17	18	15	16
4	29	33	26	28	20	20	10	15	16	18	20	24	16	21
5	8	10	8	9	–	9	–	–	–	9	11	13	9	15
Gentamicin	33	34	34	36	31	32	31	32	30	31	nd	nd	nd	nd
Fluconazole (2.0 mg ml^{-1})	–	–	–	–	–	–	–	–	–	–	30		12	

[a]Diameter of a ditch for substances, 7 mm; –, non-inhibition; nd, not determined.

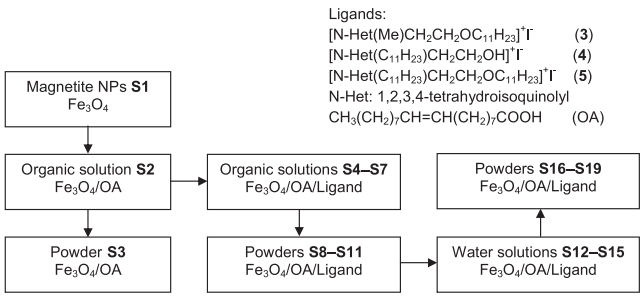
of the desired iron oxide magnetic nanostructures, which was carried out according to the consecutive reactions depicted in Scheme 2.

Preparation of samples **S4–S19** of mixed covered magnetite NPs is outlined in Scheme 3 and involves the following steps: (a) preparation of powdery sample **S1** of magnetite (Fe_3O_4) NPs, (b) treating the prepared magnetite NPs **S1** with the first surfactant, namely OA, in toluene to obtain MFs **S2** and corresponding powdery samples **S3** (Fe_3O_4/OA), (c) preparation of organic solutions **S4–S7** and corresponding powdery samples **S8–S11** of mixed covered NPs from MF **S2**, which were further used (d) to produce corresponding aqueous magnetic solutions **S12–S15** and water-soluble powders **S16–S19** (Fe_3O_4/OA/**3–5** or OA).

MFs **S2a** and **S2b** (Fe_3O_4/OA) were obtained using wet synthesis by precipitation from an aqueous solution of ferrous and ferric salts, Fe(II) sulfate and Fe(III) chloride, with excess sodium hydroxide, and followed by reaction of the prepared magnetite NPs with OA in toluene. NPs as powdery samples were isolated for physicochemical characterization. The results are reproducible when keeping the same reaction conditions, and magnetic NPs do not differ much by ratio of components determined in the corresponding **S3a** and **S3b**.

The residue **S3c**, isolated from MF **S2c**, which was prepared using sonication procedure, has slightly larger particles than the residue **S3b**, obtained from MF **S2b**, and higher iron oxide content. Nevertheless, it can be noted that ultrasound application does not significantly affect the size of the resulting NPs.

We did not succeed in attempts to obtain MF by changing the reaction procedure for shaking in toluene under heating (**S2d**): OA did not interact with magnetite in sufficient amount, its content was low (Fe_3O_4:OA = 30:1).



Scheme 3. Preparation of magnetite-based samples **S2–S19**.

Magnetic NPs **S8–S11** (Fe_3O_4/OA/**3–5** or OA), which differ in the nature of the second surfactant and initial MF used for their preparation, were obtained as powdery samples by a series of consecutive procedures: shaking of MFs **S2a**, **S2b** or **S2c** with ligands **3–5** or additional amount of OA, followed by sedimentation and drying.Powders **S8–S11** were treated with water in order to produce corresponding aqueous magnetic solutions **S12–S15** and water-soluble powders **S16–S19**, as a result of subsequent solvent evaporation. MF **S2a** was used as the initial MF for preparation of the series of samples **S4a**, **S8a**, **S12a** and **S16a** containing ligand **3** and **S5a**, **S9a**, **S13a** and **S17a** containing ligand **4**. MF **S2b** was used for the preparation of the series **S4b**, **S8b**, **S12b** and **S16b** containing ligand **3**, series **S5b**, **S9b**, **S13b** and **S17b** containing ligand **4**, series **S6b**, **S10b**, **S14b** and **S18b** containing ligand **5** and the series **S7b**, **S11b**, **S15b** and **S19b** containing OA. MF **S2c** was used for the preparation of samples **S4c**, **S8c**, **S12c** and **S16c** containing ligand **3**. The samples obtained were characterized at the stage of their preparation and/or isolation by various physicochemical methods.

X-ray (line profile broadening) diffraction analysis of the magnetite powder **S1** was employed for core size determination. Figure 2 shows the powder X-ray diffraction pattern of the synthesized particles with Miller indices and profile fitting results. The product (sample) is the cubic phase of Fe_3O_4, and there are no other phases in the sample. The whole powder pattern fitting method was used for size–strain analysis of magnetite particles. We applied the profile

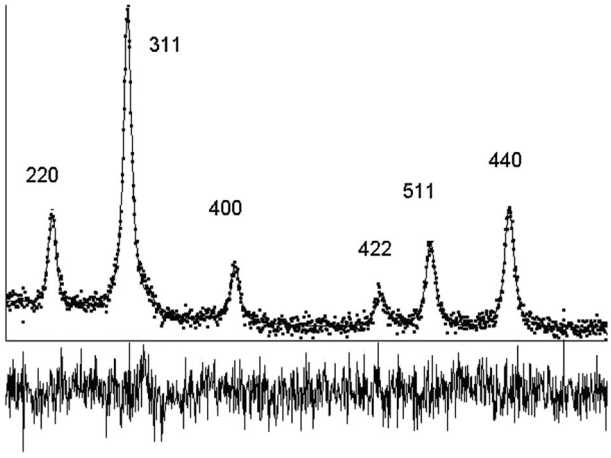


Figure 2. Powder X-ray diffraction pattern with Miller indices and profile fitting results of magnetite sample **S1**.

Table 2. Physicochemical properties of powdery samples **S1** and **S3**, **S8**, **S9** isolated from corresponding organic MFs

Sample	Content (mol/mol)	Magnetization, σ_{10kOe} (emu g^{-1})	Superparamagnetic saturation, σ_s (emu g^{-1})	Magnetite concentration, C (%)	Magnetite core size (nm)		
					d	d_{min}[a]	d_{max}[a]
S1	Fe_3O_4	53.4	50.8	55.2	10.5	7.1	14.1
S3a	Fe_3O_4/OA (1.6:1)	15.6	15.2	16.5	6.4	3.5	8.7
S3b	Fe_3O_4/OA (1.4:1)	23.6	29.7	32.3	6.6	4.1	9.2
S3c	Fe_3O_4/OA (5.7:1)	27.0	26.2	28.5	9.0	5.7	12.9
S8a	Fe_3O_4/OA/ **3** (1.6:1.0:≤0.1)	15.8	15.8	16.7	6.3	3.5	8.6
S9a	Fe_3O_4/OA/ **4** (1.6:1.0:≤0.1)	16.1	15.6	17.0	6.3	3.5	8.6

[a]At level 0.5 from distribution density maximum.

Table 3. Physicochemical characterization and yield of water-soluble nanoparticles **S16–S19**

Sample	Composition (molar ratio)	σ (emu g^{-1})[a]	C (%)[a]	Size (nm)[b]	Yield (%)
S16a	Fe_3O_4/OA/**3** (1.6:1:0.1)	4.76	6.80	6.5	—
S16b	Fe_3O_4/OA/**3** (1.4:1:0.5)	6.67	9.44	6.5	29
S16c	Fe_3O_4/OA/**3** (5.7:1:nd)[c]	10.25	10.00	10.5	6
S17a	Fe_3O_4/OA/**4** (1.6:1:0.1)	3.51	5.32	6.9	—
S17b	Fe_3O_4/OA/**4** (1.4:1:1.5)	0.118	0.176	7.9	25
S18b	Fe_3O_4/OA/**5** (1.4:1:0.7)	2.93	3.57	6.2	19
S19b	Fe_3O_4/OA/OA (1.4:1:nd)[c]	1.79	2.14	6.2	20

[a]Calculated according to the corresponding data for **S12–S15**.
[b]Determined in water solutions **S12–S15**.
[c]nd (not determined)

fitting program MarqX in the wppf3 mode including the convolution with the instrumental component and refined the model to R_{wp} = 0.9%. The trace below the X-ray pattern in Fig. 2 shows the difference between experimental and modelled data. To evaluate the size and strain effects on line broadening, we used a Williamson–Hall plot. The slope of the Williamson–Hall plot indicates negligible microstrain. Inverse to the intercept provides the average domain size of the particles of 110Å. We also calculated domain size using the LLS modelling method. This method is best suited for spherical NPs. According to the LLS modelling method, the average particle size is 90Å. The values obtained using the two methods suggest that most particles are homogenous in size and exhibit close to cubic shape.

Magnetogranulometry was used to investigate magnetic properties and determine the iron oxide magnetic core diameter of the prepared NPs. The data obtained using magnetogranulometry for the various powdery samples (**S1**, **S3**, **S8** and **S9**) are presented in Table 2. The values of magnetization for powders **S8a** and **S9a**, isolated from organic solutions of NPs, containing two different surfactants, OA and ligands **3** or **4**, and prepared from the same initial MF, are almost the same – about 16 emu g^{-1} – as is the size of the magnetic core at about 6 nm. The difference between the values for magnetite NPs obtained using X-ray diffraction analysis and using magnetogranulometry is found to be insignificant.

Magnetite concentration and particle size in the samples were calculated from the data obtained for full spontaneous magnetization and magnetic moment of magnetite particles. To analyse the weak magnetic samples, the matrix magnetization was subtracted from full magnetization.

The yield of water-soluble powdery samples **S16–S19**, bearing *O*-, *N*- and *O*,*N*-bis-undecylated ligands **3–5**, was determined from the percentage of the amount of mixed covered powders obtained from organic solutions (Table 3). It is in the range 19–29% for samples obtained from MFs **S2a** and **S2b** with a lower content of magnetite, and is less (6%) for the sample obtained from MF **S2c** with higher magnetite content.

Table 4. Size of micelles for samples in organic solution determined by DLS measurements (d, nm)

Fe_3O_4/OA	Fe_3O_4/OA/**3**	Fe_3O_4/OA/**4**	Fe_3O_4/OA/**5**
S2a – 13.5	**S4a** – 13.1	**S5a** – 13.4	**S6b** – 18.91
S2b – 20.91	**S4c** – 33.07	**S5b** – 19.42	
S2c – 31.39			
S2d – 35.92			
S7b – 21.50			

Table 5. Size of micelles for samples in water determined by DLS measurements (d, nm)

Sample	Composition	Molar ratio	d (nm)
S12b	Fe_3O_4/OA/**3**	1.4:1.0:0.5	248
S12c	Fe_3O_4/OA/**3**	5.7:1.0:nd[a]	131.2
S14b	Fe_3O_4/OA/**5**	1.4:1.0:0.7	431.1
S15b	Fe_3O_4/OA/OA	1.4:1:nd[a]	304

[a]nd (not determined)

Table 6. *In vitro* antibacterial and antifungal activity data of water solutions **S12–S15**

Sample (ligand)	Diameter of zones showing complete inhibition of growth (mm)[a]													
	SA		BC		EC		PA		PM		CA		AN	
	C_1	C_2	C_1	C_2	C_1	C_2	C_1	C_2	C_1	C_2	C_1	C_2	C_1	C_2
S12a[b] (**3**)	8	12	10	11	–	8	–	–	8	8	–	11	–	10
S12b (**3**)	17	20	10	13	–	8	–	–	–	8	–	–	nd	
S12c (**3**)	9	9	–	8	–	–	–	–	–	–	–	–	nd	
S13a[c] (**4**)	9	8	8	8	–	–	–	–	–	8	–	9	–	8
S13b (**4**)	12	12	8	8	–	–	–	–	–	–	–	–	nd	
S14b (**5**)	–	–	–	–	–	–	–	–	–	–	–	–	nd	
S15b (OA)	–	–	–	–	–	–	–	–	–	–	–	–	nd	
K_1	30	32	34	36	27	30	31	32	28	31	–	–	–	–
K_2	–	–	–	–	–	–	–	–	–	–	10		11	

[a]Diameter of a ditch for substances, 7 mm; C_1, concentration of 0.78 mg ml^{-1}; C_2, concentration of 1.57 mg ml^{-1}; –, non-inhibition; nd, not determined; K_1, gentamicin (concentration of 5 and 10 mg ml^{-1} for each test correspondingly); K_2, fluconazole (2.0 mg ml^{-1}).
[b]Concentration of 1.03 and 1.75 mg ml^{-1} for each test correspondingly.
[c]Concentration of 0.94 and 1.60 mg ml^{-1} for each test correspondingly.

DLS is used for measuring the size of colloidal particles in carrier liquids. The results for various samples in toluene and water are presented in Tables 4 and 5, respectively. The addition of the second biologically active surfactant, ligands **3–5** or OA, to the initial MF **S2** does not have much of an effect on the size of obtained micelles, which is in the range 13–36 nm. The size of micelles in water solutions is in the range 131–431 nm and depends upon NP composition. Examples of size distribution of micelles in the initial MF **S2** and in organic solution **S4** of mixed covered NPs determined using DLS are presented in Fig. S3 (supporting information).

Water MFs **S12–S15** were screened for antimicrobial activity (Table 6). Antimicrobial activity of magnetite-based samples containing amphiphilic derivatives of heterocyclic choline analogues was evaluated at concentrations of 0.78–1.03 and 1.57–1.75 mg ml^{-1} against Gram-positive and Gram-negative bacterial strains and fungi. The results show that samples **S12** (Fe_3O_4/OA/**3**) and **S13** (Fe_3O_4/OA/**4**), containing two different biologically active surfactants (OA and ligands **3** or **4**), exhibit greater antibacterial activity against Gram-positive strains and thereby retain the previously detected trend for free ligands. Compositions **S12a** and **S13a** exhibit activity against fungal strains CA and AN. Sample **S15b**, containing only OA, does not show antimicrobial activity. For the most active composition **S12b** the MIC against SA is determined as 16 μg ml^{-1}.

Conclusions

New water-soluble mixed-covered magnetite NPs precoated with OA and bearing antimicrobial N-heterocyclic choline derivatives have been obtained. The values of magnetite nanoparticle size of about 10 nm, determined using X-ray diffraction analysis and magnetogranulometry, are in good agreement.

Water MFs **S12** and **S13** based on magnetite NPs with immobilized N-heterocyclic ligands (Fe_3O_4/OA/**3** or **4**) exhibited antimicrobial properties mostly against Gram-positive strains. The most active composition **S12b** exhibited high antibacterial activity against SA with MIC value of 16 μg ml^{-1}. Compositions **S12a** and **S13a** were potent against fungal strains CA and AN.

It has been demonstrated that the obtained water MFs have superparamagnetic properties and affect microbial cell lines, retaining the type of activity inherent to the second surfactant.

The procedure developed for synthesis of mixed coated magnetic NPs bearing different types of choline derivatives is a promising route for the preparation of similar self-assembled nanostructures bearing amphiphilic biologically active compounds.

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