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Magnetic fluid poly(ethylene glycol) with moderate anticancer activity

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Magnetic fluid containing poly(ethylene glycol) with moderate anticancer activity

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Abstract

Poly(ethylene glycol) (PEG) was used to improve biocompatibility of a magnetic fluid – magnetite (Fe_3O_4) stabilized by sodium oleate. Magnetic measurements of the prepared sample confirmed superparamagnetic behaviour at room temperature. Samples were characterized using different techniques e.g. electron microscopy, small-angle neutron scattering and photon cross correlation spectroscopy. From the *in vitro* toxicity tests it was found that a magnetic fluid containing PEG (MFPEG) partially inhibited the growth of B16 cells at the highest tested dose (2.1 mg/ml of Fe_3O_4 in MFPEG). MFPEG seems to be acceptable for the application in therapy as a safe component of drug carriers. © 2009 Published by Elsevier B.V.

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Keywords: Magnetic fluid; poly(ethylene glycol); LD_{50}; biocompatibility; *in vivo* test; *in vitro* test

1. Introduction

Water based magnetic fluids have found application in a variety of fields in biotechnology and medicine (e.g. cell separations, diagnostic magnetic resonance imaging, hyperthermia, magnetic drug targeting, etc.) [Ling]. The coating of the nanoparticles is one of the most important factors responsible for their compatibility in the organism. The surface of ultrafine magnetic particles can be covered with molecules with different end groups. For our purpose poly(ethylene glycol) (PEG) was chosen because it is non-immunogenic, non-toxic, non-antigenic, biocompatible and soluble in water and organic solvents [Zal]. Highly insoluble anticancer agents can be attached to PEG, so the solubility of the modified drug will exceed that of the original drug, increasing possibility of more effective drug delivery [Green]. The aim of our work was to prepare a stable magnetic fluid by surface modification with oleate and PEG for proposed use in medicine, characterize its properties and perform *in vitro* cytotoxicit tests.

2. Materials and methods

2.1. Reagents

Ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), ferrous sulphate heptahydrate ($FeSO_4 \cdot 7H_2O$) and Poly(ethylene glycol) with M_W = 1 000 was supplied by Sigma-Aldrich Ltd. Ammonium hydroxide NH_4OH and sodium oleate were obtained from Riedel-de Haën. Deuterium oxide (D_2O) was provided by Sigma-Aldrich.

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2.2. Synthesis of magnetic fluid

The co-precipitation method of ferric and ferrous salts in an alkali aqueous medium was used to prepare spherical magnetite particles. In a typical synthesis aqueous solution of Fe^{3+} and Fe^{2+} in a molar ratio 2 : 1 was prepared by dissolving in deionised water. To this mixture of Fe^{3+} and Fe^{2+} an excess of hydroxide ions was added at room temperature, under vigorous stirring. Black precipitate of magnetite nanoparticles was immediately formed. After washing the precipitate by magnetic decantation and heating up to 50 °C, the surfactant sodium oleate ($C_{17}H_{33}COONa$) was added to the mixture to prevent agglomeration of the particles. This mixture was stirred under heating until the boiling point was reached. Magnetite particles stabilized by oleate bilayer were dispersed in water. Agglomerates were removed by centrifugation (9000 RPM for 30 minutes).

Finally, PEG was used to improve biocompatibility of the prepared magnetic fluid. PEG with a molecular weight 1 kDa, dissolved in water, was added to magnetic fluid at 50 °C at a given weight ratio PEG/Fe_3O_4. The most important ratio was 0.25 (MFPEG 0.25). The mixture was stirred for half an hour. A magnetic fluid – magnetite particles coated with sodium oleate and PEG – was formed in this way. An important fact to be mentioned is that MFPEG 0.25 (for the biological experiments) was prepared using a mechanical mixer while some other samples (e. g. MFPEG 5) were incubated in a horizontal shaker during preparation.

2.3. Complementary characterizations

2.4. TEM

Examination of the prepared magnetic fluids was done using transmission electron microscopy (TEM) Tesla BS 500 microscope normally operated at 90 kV and 80 000×magnification by the replication technique. A drop of magnetic fluid sample diluted in water was deposited on the 400 mesh copper grid and air dried before the picture was taken.

2.5. SEM

Scanning electron microscopy (SEM, JEOL 7000F microscope) was used to observe the morphology and microstructure of MFPEG). The colloidal dispersion was first diluted (typically 10^6-fold dilution), then one droplet was deposited onto an aluminium grid and dried under vacuum. After sputtering with carbon, the sample was observed.

2.6. PCCS

The particle size distribution of the prepared samples were also measured by photon cross correlation spectroscopy technique (PCCS, Nanophox, Sympatec GmbH, Germany). The samples were placed in the sample holder at least 5 min before starting the measurement. From each sample, three cross correlation functions were measured over periods of at least 600 s. The laser intensity and cuvette position were adjusted to ensure an average count rate at the detectors of 300 kcps.

2.7. SQUID (Superconducting quantum interference device) magnetometer

Magnetic properties of the prepared samples were studied by SQUID magnetometer MPMS XL-5 (Magnetic properties measuring system) which supplied magnetic fields with maximum intensity $\mu_0 H$ = 5 T and at temperature 290 K.

2.8. DSC

Thermal properties were measured using a Perkin Elmer DSC 7 calorimeter with a heating rate of 10 °C min^{-1}.

2.9. Stability

Colloidal stability of MFPEG in the presence of added electrolyte (at constant pH = 6) was observed visually.

2.10. ATR-FTIR spectroscopy

Attenuated total reflectance - Fourier transform infrared (ATR-FTIR) spectroscopy was applied to prove the presence of iron oxide, PEG, and oleate in MFPEG and to try to reveal intermolecular interactions within. Lyophilised or solid MF, MFPEG, PEG, and sodium oleate spectra were measured. The spectra were collected using an FTLA2000-100 instrument (from ABB) acquiring 32 scans for each specimen at a nominal resolution of 4 cm^{-1}. Absorbance was measured either at 37 °C using a diamond internal reflection element equipped with a temperature controller (MKII Golden Gate from Specac) or at ambient temperature using a ZnSe element (Silver Gate, Specac).

2.11. SANS

The samples were studied by small-angle neutron scattering (SANS) with contrast variation at the SANS-1 instrument, GKSS, Germany.

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2.12. Biological experiments

In vitro cytotoxicity of MFPEG 0.25 was evaluated using B16 melanoma cells.

3. Results and discussion

3.1. Magnetic and complementary characterization

The samples magnetite particles coated by bilayer of sodium oleate (MF) and MFPEG 0.25 were characterized by SQUID magnetometry. The magnetic measurements confirmed in both cases the superparamagnetic behaviour at room temperature. Saturation magnetization of the MFPEG 0.25 was 7.6 emu/g while the prepared MFPEG 0.25 contained 0.1 g Fe_3O_4 per 1 ml of magnetic fluid.

Transmission electron microscopy showed nearly spherical shape of the magnetite core of the prepared magnetic fluids MF and MFPEG 0.25 with diameters of magnetite cores in range from 4 to 11 nm (Fig. 1 a, b).

Morphology of the coated magnetic particles in MFPEG 0.25 was studied by scanning electron microscopy (Fig. 2). The surface was primarily smooth. The mean diameter of coated particles determined by this method was approximately 73 nm.

The hydrodynamic particle size distribution of the prepared samples was measured by photon cross correlation spectroscopy technique (PCCS, Fig. 3). The mean hydrodynamic particle diameters were D_H(MF) = 65 nm and D_H(MFPEG 0.25) = 76 nm. ?!

3.2. Thermal analysis

In order to verify the coating formation on the surface of magnetic particles differential scanning calorimetry (DSC) for pure PEG, lyophilised MF (magnetic particles coated by sodium oleate), MFPEGs with different PEG to Fe_3O_4 ratios, and physical mixture of PEG and lyophilised MF, were carried out (Fig. 4). The thermograms in the temperature range from 25 to 90 °C were recorded at heating rate 10 °C/min. The melting temperature of pure PEG was about 47 °C and the temperature is not changed for the physical mixture consisting of the same components of PEG and MF. However, in the system of adsorbed PEG on MF, the melting temperature was shifted to lower temperature of about 41 °C. In the case of higher amount (above weight ratio PEG/Fe_3O_4 > 10) of PEG in MF, not all PEG was adsorbed on magnetic particles and as a consequence, the melting peak was splitted indicating a presence of non-adsorbed PEG on magnetic particles (data not shown). By comparison of the thermograms, it is evident that the prepared oleate-magnetite particles were coated by PEG.

3.3. ATR-FTIR spectra

The collected spectra are shown in Fig. 5IR. The PEG/magnetite ratio, important for the ATR-FTIR results, is indicated within the sample designation. The spectrum of MFPEG 5 (A) is described in more details below. The absorption at 2918 - 2858 cm^{-1} corresponded to stretching C-H (ν(C-H)) vibrations of PEG and oleate. The asymmetric stretching of carboxylate anion ($\nu_{as}(COO^-)$) had a maximum at 1564 cm^{-1}, which is a 4 cm^{-1} higher wavenumber compared to the $\nu_{as}(COO^-)$ observed for the initial MF used for the preparation of MFPEG 5 (C). The shape of this band was also changed. The presence of PEG was obvious in the region ca. 1360 - 840 cm^{-1}. The strongest band at 1103 cm^{-1} was ascribed to ν(C-O) vibration of the polyether. The shoulder at 1061 cm^{-1} was reduced compared to the spectrum of PEG (B). Also, a maximum emerged at 960 next to the 947 cm^{-1} maximum, possibly due to a strengthening of shoulder of the original PEG band at 945 cm^{-1}. The broad band around 561 cm^{-1} of ν(Fe-O) proved the presence of iron oxide. In transmission spectra of magnetite, the maximum of this band is usually observed at higher wavenumbers, e. g. 580 cm^{-1}. This significant ν(Fe-O) difference was interpreted as a feature of the ATR method.

Beside the analysis of chemical composition we tried to gain information about the intermolecular interactions of the components. Particularly, the binding of oleate anions onto magnetite surface (chemisorption) and the interaction of PEG molecules with the second layer of the oleate bilayer were of interest. Liu et al. [Liu2006] showed that bands at 1590 and 1430 cm^{-1} arising from stretching vibration of adsorbed carboxylate anions can prove the chemisorption of oleate anions on magnetite. For MFPEG 5, we did not observe significant absorptions at 1590 and 1430 cm^{-1}. This is not a disagreement because our preparation involved excess sodium oleate and MFPEG 5 contained high amount of PEG. To gain some evidence of chemisorption under such conditions, spectra of solid sodium oleate and a lyophilised typical MF without PEG measured at 37 °C were investigated (Fig. 5IR - inset II). Broadening and emerging shoulders (ca. 1540 - 1475 cm^{-1}) of the band at 1556 cm^{-1}, compared to solid sodium oleate (1558 cm^{-1}), and increasing absorption at 1404 cm^{-1} corroborated the chemisorption of oleate anions on magnetite.

As mentioned above, the $\nu_{as}(COO^-)$ band was at higher wavenumber in MFPEG 5 than in its initial MF. Changes in the shape and position of $\nu_{as}(COO^-)$ were investigated at PEG/magnetite ratios below 5 too, but we did not observe such a marked change using ATR-FTIR technique. We could not prove a rule that the higher ratio the bigger wavenumber shift. But we observed differences between MFPEG samples prepared with a mixer or with shaking. According to our other, non-spectroscopic observations, structural changes resulting from PEG addition are time (ageing) dependent.

The change of the carboxylate band in MFPEG with high PEG/magnetite ratio suggests a relatively strong interaction between the molecules of PEG and the outer layer of oleate-coating, perhaps ion - dipole interaction. Dissimilarities associated with PEG absorption bands between the spectra of pure PEG and MFPEG 5 might be signs of conformational changes of PEG molecules.

3.4. SANS

The influence of PEG was studied in view of structuralization of magnetic particles by small-angle neutron scattering (SANS) with contrast variation [Avd2007] at the SANS-1 instrument, GKSS, Germany. The initial samples were dissolved with the ratio 1:3 by different mixtures of light/heavy water, thus varying the D_2O content over the interval of 0-70 % in the final fluid. The addition of PEG to an oleate-stabilized MF may cause considerable structural changes. Large (D > 100 nm) fractal-like aggregates of individual (non-aggregated) magnetic particles with the magnetite core size of 8 nm were observed at high PEG/magnetite ratio (ca. 2.5 by mass) as opposed to compact (D < 40 nm) aggregates present without added PEG [Avd2010]. At smaller PEG/magnetite ratio of 0.25 the initial aggregates change less significantly (Fig. 6), which is an indication of only partial substitution of sodium oleate with PEG on free magnetite surface. The MFPEG of the considered PEG/Fe_3O_4 ratio was interesting because of its application in the preparation of magnetic nanospheres, which carried the anticancer drug Taxol [Zav2009]. The changes in the scattering curves (Fig. 6) were analyzed in terms of the modified basic functions $\tilde{I}_c(q)$, $\tilde{I}_s(q)$, $\tilde{I}_{cs}(q)$ [Avd]. The model expression

$$I(q) = \tilde{I}_s(q) + \Delta\tilde{\rho}\tilde{I}_{cs}(q) + (\Delta\tilde{\rho})^2 \tilde{I}_c(q) \quad (1)$$

was fitted simultaneously to all curves at different modified contrast defined as

$$\Delta\tilde{\rho} = \bar{\rho}_e - \rho_s , \quad (2)$$

where $\bar{\rho}_e = \langle\rho V^2\rangle/\langle V^2\rangle$ is the averaged scattering length density (SLD), ρ, over all particles (with volume V) in the system and ρ_s is SLD of the solvent. First, $\bar{\rho}_e$ (also called the

effective match point) was found from the minimum of the forward scattering intensity (obtained by the Indirect Fourier Transform (IFT)) as a function the D_2O content in the carrier. We were mostly interested in $\tilde{I}_c(q)$ (shown in inset to Fig. 6), which is the averaged shape scattering function taking into account the type and size polydispersity. Two regions with the features of the Guinier law can be seen in $\tilde{I}_c(q)$ and are related to compact aggregates (small q-values) and micelles of free sodium oleate (large q-values). As followed from IFT, in the spherical approximation the mean size of the aggregates is 33.0(5) nm, while the maximal size exceeds 40 nm. Since SLDs of PEG and sodium oleate are close to that of light water, at 0 % of D_2O the scattering comes mainly from magnetite. From the comparison of this scattering with $\tilde{I}_c(q)$ by the IFT treatment the difference in the maximal sizes is detected. It corresponds to the thickness of the stabilizing shell, which is estimated to be 2.0(1) nm. The analysis of the second specific q-region gives the size of about 4 nm and concentration of above 1 vol. % for micelles of sodium oleate.

The comparison of MF and MFPEG 0.25 fluids shows that PEG adsorption changed only slightly the structural characteristic of the initial fluid with respect to both the aggregate structure (thickness of the stabilizing shell and the effective match point) and micelles of non-adsorbed surfactant. This indicates that PEG forms the shell around magnetite with parameters close to the initial coating by sodium oleate. Taking into account the chain length of the used polymer (about 10 nm), one can conclude that PEG adsorbs with a flat configuration on magnetite, as shown previously for other materials [Mot1994, Dij1990].

3.5. Stability

The observations of the stability of the prepared MFPEG 0.25 at the different salinity made with the naked eye are in Fig. 7. It was in good agreement with former investigations done by PCCS and turbidimetry which gave a critical aggregation concentration of 0.095 mol.dm^{-3} for MFPEG 0.25 [Zav2009]. This value may seem to be too low for biological applications as physiological saline has a concentration ca. 0.15 mol.dm^{-3} NaCl. In contrast, MFPEG 0.25 was successfully tested in an *in vivo* experiment (see below). The biocompatibility might be explained by the interaction with proteins present in blood, which may prevent coagulation of the magnetic particles. This possibility was suggested by our other observations that certain magnetic fluids can be mixed with albumin solutions of relatively high ionic strength without coagulation while mixing with albumin-free solutions causes coagulation.

3.6. Biological experiments

In vitro cytotoxicity of MFPEG 0.25 was evaluated. Influence on the growth of mouse B16 melanoma cells during three-day exposition period was observed at three dilutions of MFPEG: 2.1, 0.084, and 0.042 mg Fe_3O_4/ml - and compared with control B16 cells. After 24 h and 48 h of treatment small or no inhibition was observed. The highest observed growth inhibition, ca. 50 %, was after 72 h at conc. 2.1 mg Fe_3O_4/ml (Table 1).

In vivo toxicity of MFPEG 0.25 was studied formerly [Zav2009]. The LD_{50} value of magnetic fluid MFPEG 0.25 (originally 0.1 g Fe_3O_4/1 ml, diluted 1:1 before application) determined in male ICR mice was 396 mg Fe_3O_4/kg.

4. Conclusion

We have prepared a biocompatible magnetic fluid – magnetite particles covered with sodium oleate and poly(ethylene glycol) dispersed in water. Prepared samples exhibit superparamagnetic properties. Size distribution of the particles was measured by different

methods. Magnetic particles coating by PEG in MFPEG was confirmed by DSC measurements. The ATR-FTIR spectrum of MFPEG 5 provided recognition of PEG and the magnetic particles with the stabilising surfactant sodium oleate. ATR-FTIR spectroscopy results also supported the expected chemisorption of a first layer of oleate on iron oxide and the interaction between PEG molecules and oleate anions at high PEG/magnetite ratio could be demonstrated. In MFPEG 0.25 used for *in vivo* experiments no fractals were observed as opposed to a higher PEG/magnetite ratio. From the *in vitro* toxicity tests it was found that MFPEG inhibited the growth of B16 cells at the highest tested dose to ca. 50 %. These results seemed to be promising for using MFPEG in the field of biomedicine, as a safe component of drug carriers.

Acknowledgements

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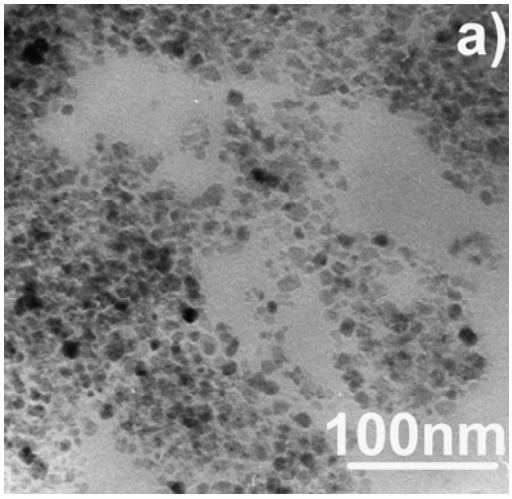

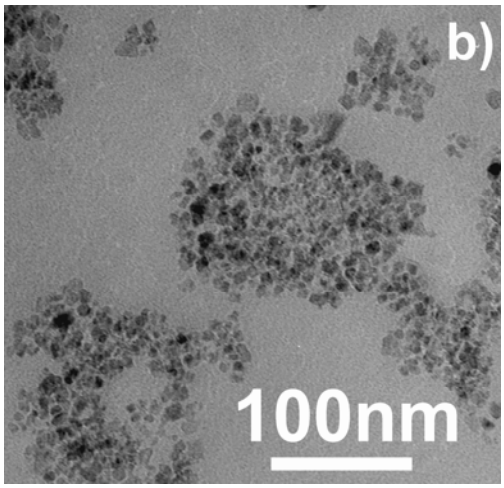

Fig. 1. TEM image of a) magnetic particles coated by sodium oleate in MF and b) magnetic particles in MFPEG 0.25.

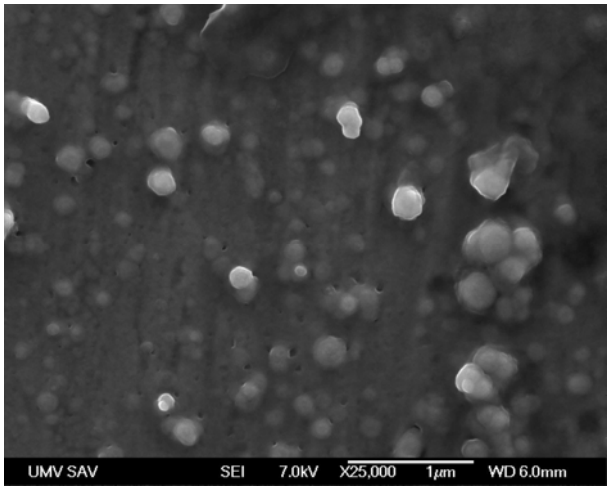

Fig. 2. SEM image of covered nanoparticles in MFPEG 0.25.

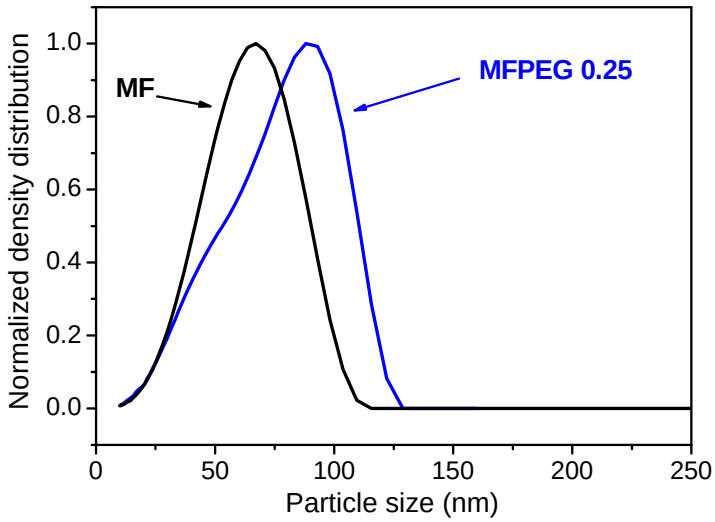

Fig. 3. Comparison of the hydrodynamic particle size distributions of MF and MFPEG 0.25 determined by PCCS.

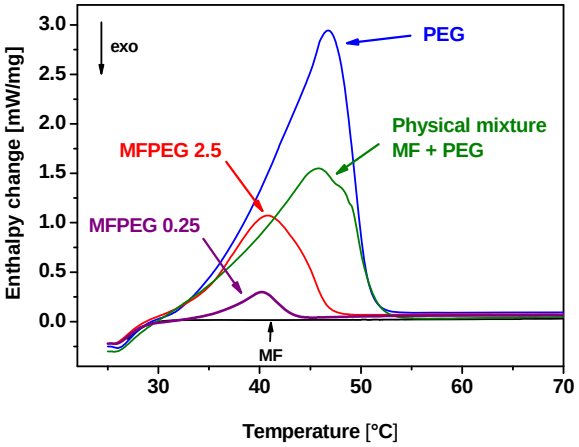

Fig. 4 DSC measurement of Pure PEG; MF - Magnetic fluid; Physical mixture of MF and PEG; MFPEG 0.25; MFPEG 2.5 - Adsorption of different amount of PEG on MF .

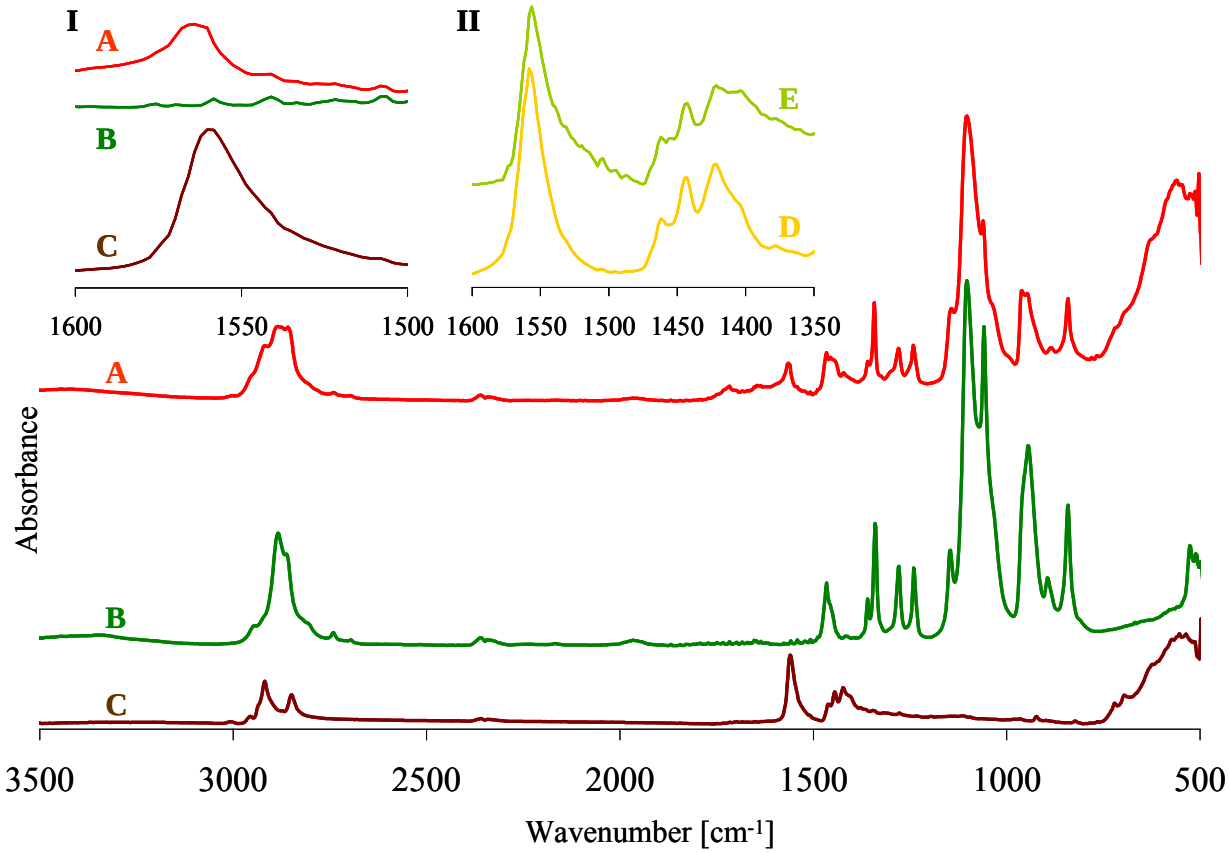

Fig. 5IR ATR-FTIR spectra of lyophilised samples MFPEG 5 (A) and its initial MF used for its preparation (C). Solid PEG (B) also shown. Inset I shows in detail the influence of PEG on oleate anions in MFPEG 5. Inset II shows spectra of solid sodium oleate (D) and a freeze dried typical oleate-stabilised MF without PEG (E). A - C collected with a ZnSe internal reflection element, D and E using diamond element. Spectra vertically shifted.

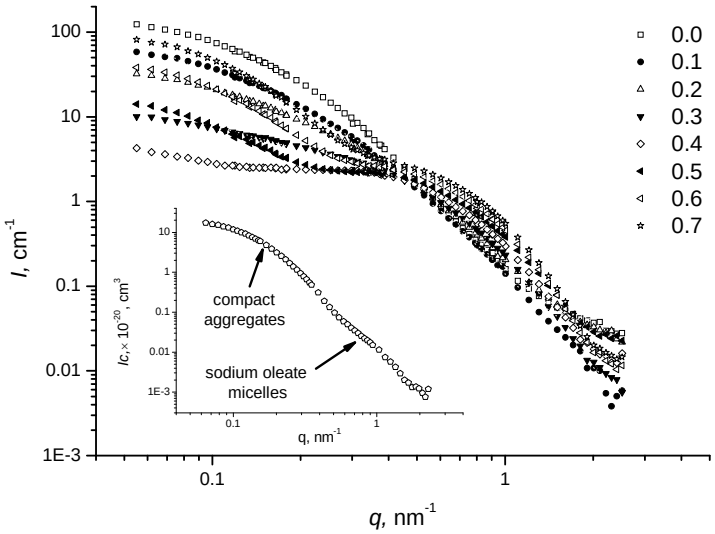

Fig 6. SANS Contrast variation of MFPEG. $I(q)$ at D_2O contents (volume fractions in solvent) from 0 to 0.7 (37 °C). Inset shows the averaged shape scattering function with indicated scattering levels.

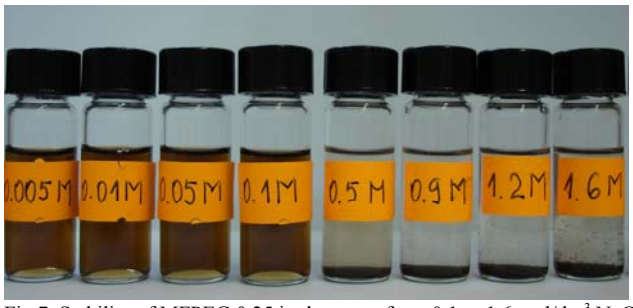

Fig.7. Stability of MFPEG 0.25 in the range from 0.1 to 1.6 mol/dm^3 NaCl concentration pH=6 (at ambient temperature).

Table 1
Influence of MFPEG 0.25 on growth of B16 melanoma cells

Dilution of MFPEG 0.25 concentration of Fe_3O_4	% inhibition		
	24h	48h	72h
0.042 mg/ml	8.8	0	0
0.084 mg/ml	7.3	0	36.2
2.1 mg/ml	21.9	10.3	56.1