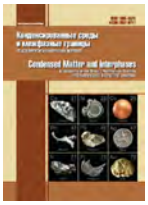

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Synthesis and experimental study of liquid dispersions of magnetic fluorescent polystyrene microspheres

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Abstract

Multiplex microsphere-based immunofluorescence assay is a reliable, accurate, and highly sensitive method for the detection of various biomolecules. However, for the moment, the wide application of the method in clinical practice is prevented by the high cost of reagents for analysis - magnetic spectrally encoded microspheres. Therefore, an urgent task is the development of new methods for the synthesis of microspheres with the required properties. The aim of this study was the creation of new magnetic fluorescent microspheres suitable for use in multiplex immunoassay.

Samples of magnetic fluorescent polystyrene microspheres were synthesized by dispersion polymerization and two-stage swelling methods. Experimental studies of geometric parameters, fluorescence, magnetic properties of the synthesized microspheres have been carried out.

The results of the studies have shown that microspheres synthesized by dispersion polymerization are promising for the use in immunofluorescence analysis. The obtained results can be used for the development of new diagnostic multiplex test systems based on spectrally encoded microspheres.

Keywords: Immunofluorescence assay, Planar immunoassay, Microspheres, Fluorescence, Dispersion polymerization, Two-stage swelling

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1. Introduction

Now, fluorescent microspheres are widely used in various methods of immunoassay. Immunofluorescence analysis based on microspheres has a number of important advantages, such as the ability to simultaneously detect several analytes in one sample (multiplicity), a small sample volume required for analysis, as well as high accuracy and sensitivity at a high analysis speed.

Multiplex microsphere-based analysis methods are widely used in scientific research, medicine, biology, and immunology. These methods are used for the detection of nucleic acids [1], the detection of carcinogens and toxins in food [2, 3], the detection of organophosphate pesticides, causing environmental pollution [4], for the diagnosing of tumour markers [5, 6] and various infections [7], for the detection of coronavirus infection COVID-19 [8].

Multiplexity in modern analysis methods is usually achieved through the use of spectrally-encoded microspheres [9]. Spectral coding of microspheres is used for the creation of different types of microspheres to which the analysed analytes will correspond, and is carried out by staining the microspheres with various fluorescent dyes in different concentrations. Thus, for example, the use of a combination of three dyes in different concentrations allows encoding up to 500 types of microspheres [10].

Most methods of multiplex immunoassay are based on a specific antigen-antibody reaction. Immunocomplexes are formed on the surface of optically encoded polymeric microspheres. For example, the so-called "sandwich" method involves the formation of immunocomplexes "primary antibody - detectable antigen - detecting antibody – fluorescent label" on the surface of microspheres (Fig. 1).

The reading of fluorescence signals for identification of microspheres and detection of a fluorescent label during a multiplex immunoassay can be performed by different methods.

A widely used approach is based on flow cytometry, in which particles are analysed in the flow one by one [11]. In the flow cell, the particles are irradiated with laser radiation, the signals of light scattering and fluorescence from each particle are recorded using detectors based on photomultiplier tubes (PMT) or avalanche photodiodes (APD).

Over recent years, flow cytometry has been replaced by a new method - planar immunoassay based on fluorescent magnetic microspheres [12]. In this method, particles are immobilized in one plane using a magnet, after which fluorescence is excited using LED and recorded using a CCD (charge-coupled device) or a CMOS (complementary metal-oxide-semiconductor) camera using the necessary light filters (Fig. 2).

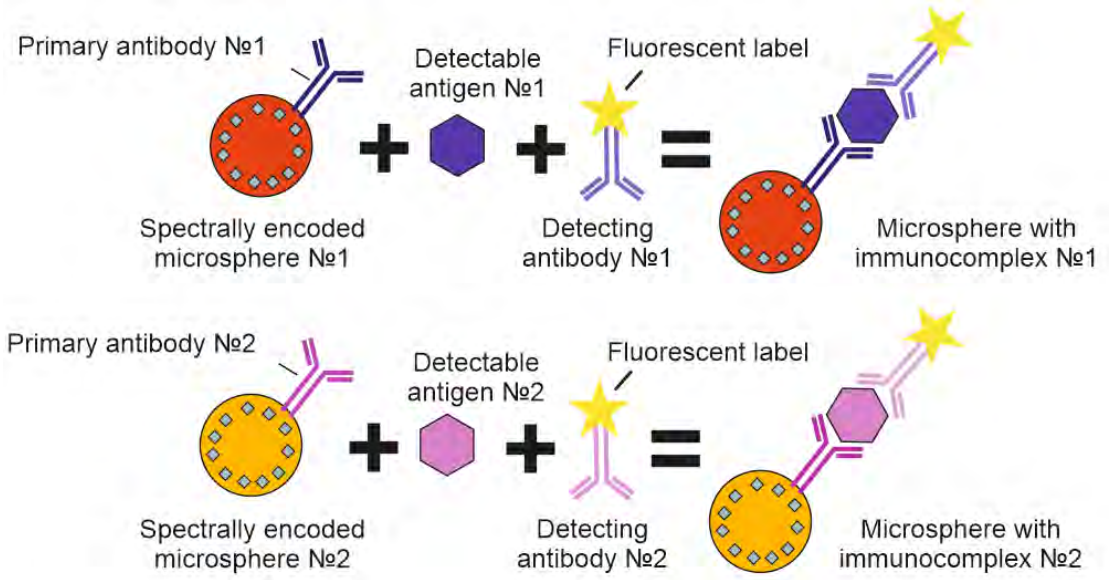


Fig. 1. Formation of immunocomplexes on the surface of microspheres in the multiplex immunoassay method

Analysis of the obtained images in different fluorescence channels allows identifying the spectrally encoded microspheres. After identification of the microspheres, the signal from the fluorescent label is recorded in the same way using an appropriate filter. In this case, the presence or absence of fluorescence from a certain type of microsphere indicates the presence or absence of the corresponding analyte in the sample. In addition, the value of the fluorescence intensity can be used to estimate the concentration of the analyte in the sample, since the fluorescence intensity depends on the amount of the analyte bound to the microsphere [13].

Instruments based on the method of planar immunoassay are more affordable, compact and reliable in comparison with analysers based on flow cytometry [14].

Multiplex analysis based on magnetic spectrally encoded microspheres is a reliable, accurate, and highly sensitive method [15]. However, currently, the wide application of the method in clinical practice is prevented by the high cost of reagents for analysis - magnetic spectrally encoded microspheres, due primarily to the closed technology.

The aim of this study was the creation of new magnetic fluorescent microspheres suitable for use in multiplex immunoassay.

Magnetic fluorescent polystyrene microspheres samples were synthesized by the methods of dispersive polymerization and two-stage swelling within the framework of this study. Experimental studies of geometric parameters, fluorescence, magnetic properties were performed in order to assess the possibility and prospects of using the synthesized microspheres in multiplex immunofluorescence analysis.

2.Experimental

2.1. Reagents

The following reagents were used for the synthesis of microspheres: styrene, Pur., Russia (purified by vacuum distillation with preliminary removal of the inhibitor with an aqueous solution of NaOH); methyl methacrylate containing 10–110 ppm of 4-methoxyphenol as inhibitor (99%, ACROS Organics); divinylbenzene (80% mixture of isomers, Aldrich), 2-ethoxyethanol, P.A., Russia; tetrahydofuran, Pur., China; ethanol, 95%; toluene, P.A., "Reakhim"; Pluronic F-127, Sigma, BioReagent, CCM = 950–1000 ppm; azobisisobutyronitrile (AIBN), C.P., Russia; sodium lauryl sulphate, P.A., "Reakhim"; acetone, P.A., Russia; polyvinylpyrrolidone (mol. weight - 40,000 g/mol) "Sigma-Aldrich"; magnetic fluid in kerosene was provided by Nanocomposite LLC, average particle radius is 9 nm, particles were stabilized with oleic acid; deionized water, 13–15 MOhm cm.

2.2. Synthesis of microspheres

The synthesis of polymer particles is based on the method of dispersion polymerization (sample No.1) [16-18] and the method of two-stage swelling (sample No.2) [19, 20]. The main requirement for polymer particles is based on the use of "hard" solvents (for example, toluene, tetrahydofuran) at the stage of introducing magnetic particles and staining with fluorescent dyes. Thus, the particles must have a high

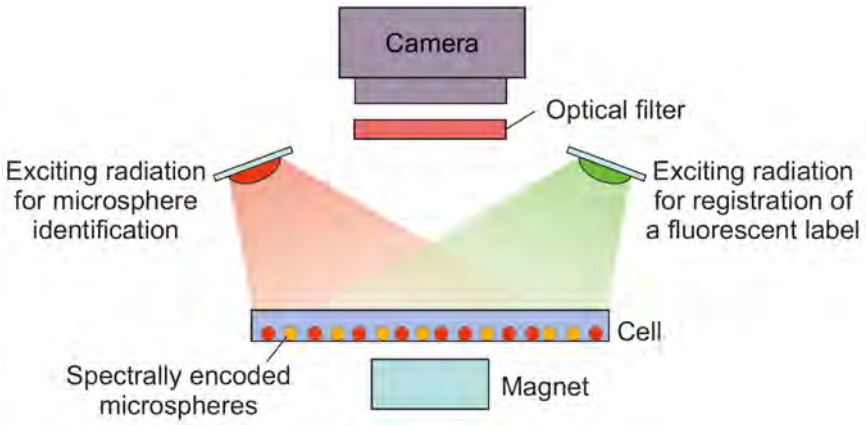


Fig. 2. Planar immunoassay based on fluorescent magnetic microspheres

crosslinking density in order to avoid dissolution and excessive swelling.

2.2.1. Synthesis of sample No.1 by dispersion polymerization method

The synthesis was carried out in a temperature-controlled reactor at a temperature of 74 °C, equipped with a rotary stirrer, a reflux condenser, a funnel for supplying reagents, and a capillary for nitrogen blow. The stirrer speed was 150 rpm. Ethanol was used as the reaction medium. The following reagents were mixed separately on a magnetic stirrer: 15 ml of styrene and 0.41 g of AIBN. 0.85 ml of divinylbenzene-80 in 10 ml of 2-ethoxyethanol was introduced 10 min after the initiation. The SEM of the obtained particles is shown in Fig. 3a.

2.2.2. Synthesis of sample No.2 by two-stage swelling method

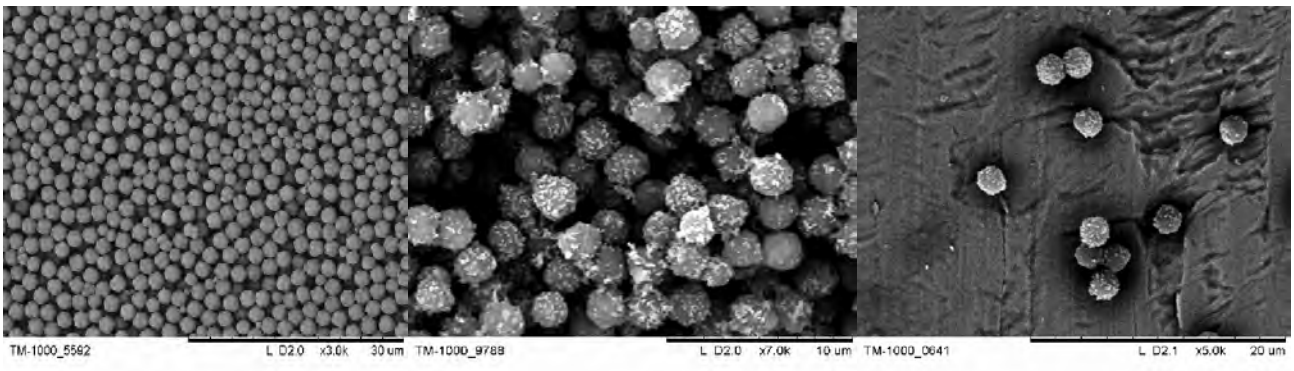
First, 1 g of the initial polymer particles, preliminarily obtained by dispersion polymerization (Fig.4a), with a molecular weight of about 10,000 Da was dispersed in 50 ml of an aqueous solution of sodium lauryl sulphate (0.25 wt. %) using an ultrasonic bath for 30 min. Then an emulsion containing 10 ml of acetone in 50 ml of 0.25 wt. % sodium lauryl sulphate solution was added to the solution, and the mixture was left stirring for 12 hours at a temperature of 25 °C for the first stage of swelling.

At the next stage, a monomer emulsion, consisting of 1 ml of styrene, 1 ml of methyl methacrylate, 2 ml of a crosslinking agent (divinylbenzene-80) with a dissolved initiator (AIBN, 1–2 wt. % of the monomers) in 50 ml 0.25 wt. % sodium lauryl sulphate solution was added to the resulting mixture, and left for the second stage of swelling for 8-12 hours.

For the polymerization, 50 ml of 1 wt. % solution of the stabilizer polyvinylpyrrolidone-40 was added, the temperature of the mixture was raised to 70 °C and held for 10 hours. The resulting suspension was washed with water (3 times) and ethyl alcohol (3 times) for the removal of unreacted monomer and small side particles. The SEM of particles after two-stage swelling is shown in Fig. 4a.

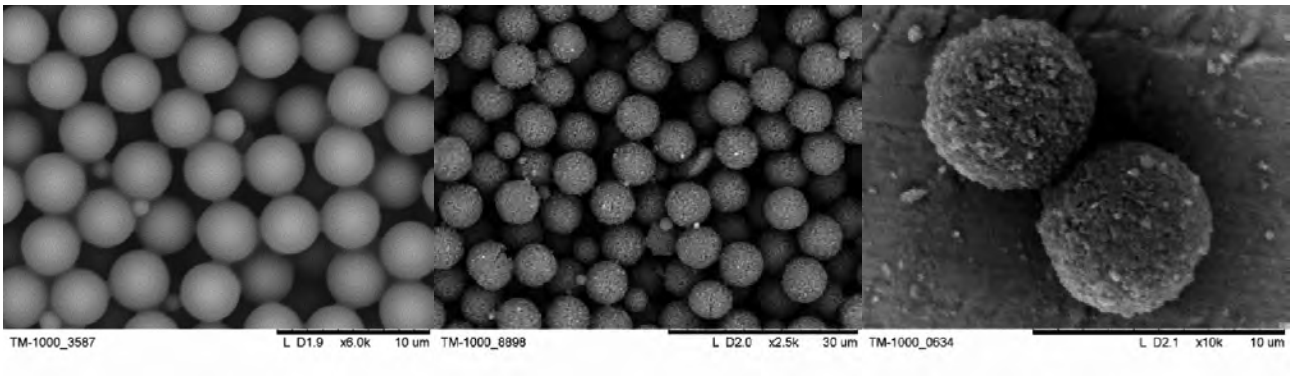
2.3. Method of introducing magnetic particles into polymer microspheres

Polymer particles were centrifuged in ethanol three times for 20 min at 907 g for sample No.1, and at 403 g for sample No.2, on a ThermoFisher Heraeus Labofuge 200 centrifuge, after which they were transferred into butanol-1 and butanol three more times with the same centrifugation parameters. Then, a magnetic fluid in toluene was added drop wise under the action of ultrasound on a 5 ml vessel in a VGT-1613QTD ultrasound bath with a volume of 1.3 L with an operating frequency of ultrasonic vibrations of 40 kHz. The particles were sonicated for at least two hours, after which they were separated by centrifugation three times for 20 min at 45 g from the magnetic particles that were not attached to the spheres. The particles were repeatedly washed with ethanol and water, separating them with permanent magnets (Nd–Fe–B) with pull off forces of 2.35, 8.62, 20.65 kg, while separating the middle fraction of particles. For the subsequent staining of the particles, they



a *b* *c*

Fig. 3. SEM images of: a) original sample No.1 obtained by dispersion polymerization; b) sample No.1 after application of magnetic particles; c) sample No.1 after application of the dye and magnetic particles



a *b* *c*

Fig. 4. SEM images of: a) original sample No.2; b) sample No.2 after two-stage swelling; c) sample No.2 after application of the dye and magnetic particles

were transferred into deionized water containing 0.5 wt. % Pluronic F-127.

2.4. The introduction of dyes into polymer microspheres

Cyanine fluorescent dyes Cy5 and Cy5.5 (Fig. 5) were introduced into particles by the diffusion method [21]. The dyes were dissolved in a tetrahydrofuran medium and added drop wise to a known amount of polymer magnetic particles dispersed in deionized water containing 0.5 wt. % Pluronic F-127. The dyes were left to diffuse into polymer spheres at 40 °C for 6 hours with constant stirring on a UT-4331S shaker. The spheres were separated with a magnet and washed with water several times, after which they were transferred to deionized water.

2.5. Research methods

The study of the geometric parameters of the samples was carried out by of optical and electron microscopy methods, as well as by the dynamic light scattering (DLS) method.

Scanning electron microscopy (SEM) images were obtained using a TM-1000 microscope (Hitachi, Japan).

The optical microscope LOMO BIOLAM M-1 (LOMO, Russia) was used in the optical microscopy experiments. ImageJ software (NIH, USA) was used for image processing and automatic analysis. The geometric parameters of at least 500 particles of each sample were measured for obtaining statistically reliable results in the course of automatic image analysis.

In the dynamic light scattering method, the investigated liquid dispersion of particles is irradiated by a laser and the scattered laser radiation is recorded. Since the particles are in continuous Brownian motion, local inhomogeneities of the refractive index appear,

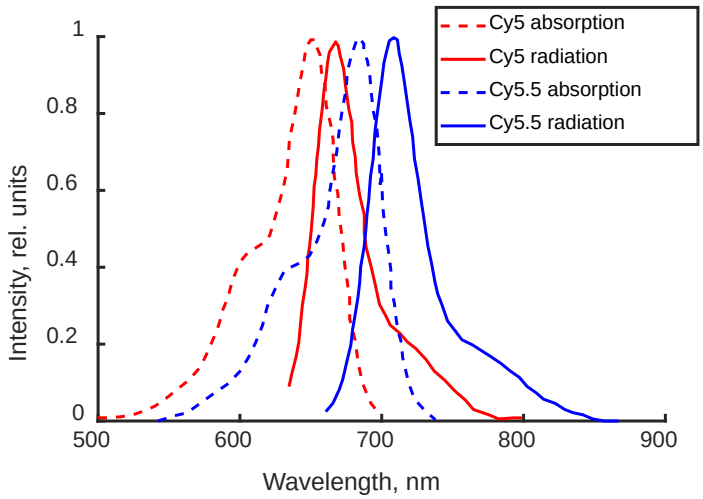


Fig. 5. Absorption and fluorescence spectra of Cy5 and Cy5.5 dyes

which, when light passes through the medium, lead to fluctuations in the intensity of the scattered light. The diffusion coefficient is measured based on the analysis of the time autocorrelation function (ACF) of the scattered light intensity. The hydrodynamic radius of the particles is then calculated using the Stokes-Einstein formula.

The DLS method was used to study synthesized samples for the presence of large particle aggregates in them. According to the theory of light scattering for small particles with radius R, with a refractive index n_1 in a liquid with a refractive index n_2 scattered radiation intensity I is equal to:

$$I = \frac{16\pi^4 R^6}{r^2 \lambda^4} \left(\frac{n^2 - 1}{n^2 + 2} \right) \psi I_0, \quad (1)$$

where r is the distance from the scattering region to the observation point, λ is the wavelength of the incident light beam, n – relative refractive index (n_2/n_1), ψ is the angle between the direction of polarization of the incident wave and the direction of scattering, I_0 – the intensity of the incident radiation, – the intensity of the scattered radiation. From formula (1) it can be seen that the intensity of the scattered light is determined by the radius of the particle raised to the sixth power. Thus, when analysing a mixture of particles of different sizes by the DLS method, the intensity of the scattered light from larger particles will be significantly higher. In this regard, the method allows registering even small concentrations of large particles in a sample of a liquid dispersion.

The DLS measurements were carried out using a Photocor Complex particle size analyser (Photocor Ltd, Russia). A laser with a wavelength of 657 nm was used for the measurements. The measurements were carried out at a temperature of 23 °C. When calculating the hydrodynamic radius of particles, the viscosity of water at a given temperature – (0.9 cP) and the refractive index of the medium (1.332) were used. The ACF was measured for a scattering angle of 30°; the ACF accumulation time was 30 seconds. For each sample at least 10 measurements were carried out.

The experimental setup was developed for obtaining fluorescent images of spectrally encoded microspheres, the scheme of the setup is shown in Fig. 6. A sample of the suspension of microspheres was placed in the optical cell on an object stage and illuminated with a laser. CPS635F laser with a wavelength of 635 nm (Thorlabs Inc., USA) was used to excite the fluorescence of cyanine dyes. For the registration of the fluorescence, bandpass filters 661/20 nm and 700/13 nm BrightLine (Semrock, USA) for Cy5 and Cy5.5, respectively, were used.

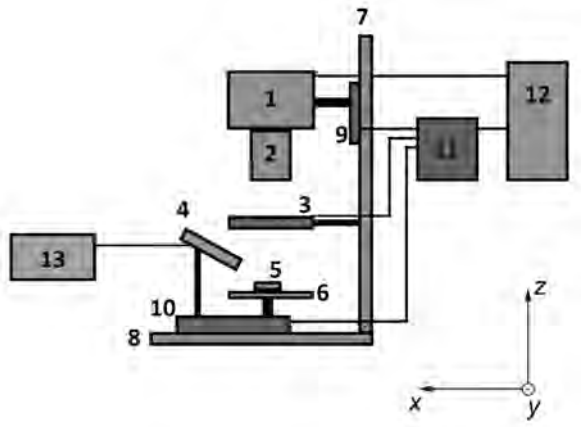


Fig. 6. Scheme of the experimental setup for fluorescence imaging of microspheres: *1* – camera, *2* – microscope lens, *3* – motorized filter wheel, *4* – laser, *5* – cell with a sample, *6* – object stage, *7*, *8* – vertical and horizontal guides, *9* – linear translator for moving the camera along the axis *z*, *10* – linear translator for moving the sample along the axes *x*, *y*, *z*, *11* – linear translator controller, *12* – PC, *13* – laser power controller

A highly sensitive Kingfisher 6MP CCD camera (Raptor Photonics, UK) and a 20x microscope objective were used to obtain fluorescent images of microspheres. The analysis of the obtained images was carried out using the ImageJ program.

Samples were also analysed in a QuattroPlex analyser (Aivok LLC, Russia) for the assessment of the magnetic properties of particles. The device is designed for multiplex immunofluorescence analysis based on magnetic spectrally encoded microspheres. During the analysis, the microspheres are immobilized in the flow cell using a magnet, after which fluorescence images are obtained in different fluorescence channels. Conclusions about the magnetic properties of the particles can be drawn based on the obtained images.

3. Results and discussion

Despite the significant laboriousness of two-stage swelling, the method has become one of the main methods for obtaining particles with a size of more than two microns [22–24]. The corresponding theoretical basis of the method was actively developed due to problems with the quality of the product obtained by an alternative method - dispersion polymerization. For the use of the particles in multiplex immunofluorescence analysis, they must withstand "hard" solvents (e.g., toluene, tetrahydrofuran, etc.), which are used in the diffusion staining method and the method of introducing magnetite nanoparticles. Particles meeting these requirements can theoretically be obtained by the method of dispersion polymerization: the polymerization is carried out in the presence of a large proportion of the cross-linking agent in relation to the basic monomer. However, a significant disadvantage of the dispersion polymerization method in the presence of a significant amount of a crosslinking agent is uncontrolled aggregation of particles and a high value of the polydispersity coefficient of the resulting particles [25]. Over recent years, it was proposed to add a crosslinking agent after the end of the nucleation of the primary particles in order to solve the above problems in the dispersion polymerization method. However, the task of determining the optimal moment of adding the crosslinking agent is not trivial due to the wide range of conditions under which synthesis is usually carried out for obtaining the desired particle size and degree of polydispersity. Moreover, it remains unclear how the time of addition of the crosslinking agent will affect the crosslinking density in the resulting particle [26]. Therefore, a comparison of the properties of particles obtained by these two methods was made for the selection of particles with the desired properties: the degree of monodispersity, the proportion of impurities of irregular shape, magnetic properties, and the variation of fluorescence brightness.

3.1. Particle size

Micrographs were obtained using optical microscopy for both microsphere samples. As a result of automatic image analysis, information was obtained on the average particle size, which was 2.2±0.7 μm for sample No.1 and 6.5±0.4 μm for sample No.2.

The optimal particle size for use in a microsphere-based immunofluorescence assay is determined by instrumental and biochemical factors. Thus, the possibility of using particles of small diameter (less than 0.5 μm) is limited by the complexity of their detection, as well as by the low degree of adsorption of the analyte associated with the small surface area of the microspheres. These factors significantly reduce the sensitivity of the analysis. At the same time, the large surface area of the microspheres (with a diameter of more than 9 μm) can cause uneven distribution of the analyte during the analysis and the high consumption of expensive reagents (first of all – antibodies). For example, magnetic polystyrene microspheres prepared by spray suspension polymerization have a narrow size distribution, but a large average diameter of about 10 μm [27].

Therefore, microspheres with sizes of 2.2 and 6.5 μm are suitable for use in immunofluorescence assays.

3.2. Particle shape

The particle shape different from spherical complicates the analysis of images in the process of immunofluorescence analysis and negatively affects its accuracy [28].

For a quantitative assessment of this parameter based on the obtained micrographs of the samples, the coefficient of roundness of particles was measured k_c:

$$k_c = 4\pi \frac{A}{P^2}, \quad (2)$$

where A is the area of the particle, and P is the length of its outer border in the image. Thus, the value $k_c = 1$ corresponds to a perfect circle, and the values k_c approaching 0 correspond to a more elongated particle shape.

The measured values of the coefficient of roundness were 0.90 ± 0.02 for sample No.1 and 0.92 ± 0.02 for sample No.2. Thus, for both synthesized samples, the difference between the particle shape and spherical shape is insignificant and will not negatively affect the analysis results.

3.3. The degree of monodispersity

Monodispersity of particles is also an important parameter. First, a high degree of monodispersity

is necessary for accurate identification of microspheres, since the fluorescence intensity of an individual microsphere depends on its size. This is especially important when microspheres are encoded with different concentrations of a fluorescent dye.

Second, the accuracy of determining the analyte concentration will depend on the degree of monodispersity, since the kinetics of the reaction on the surface of the microsphere and the fluorescence intensity of the label depend on the surface area of the particle [29].

The results of the study by optical microscopy indicate a rather high degree of monodispersity for both samples. The coefficient of variation was 7.4% for sample No.1 and 6.2% for sample No.2.

For comparison, the study [30] described microspheres synthesized by dispersion polymerization in an alcoholic medium, the coefficient of variation of the sizes of which ranged from 14.2 to 21.7%, depending on the amount of the stabilizer.

3.4. The presence of impurities and particle aggregates

The presence of particle aggregates can have a negative effect on the results of immunofluorescence analysis, since such particles during the analysis will either provide a false signal of fluorescence or will not be taken into account in the analysis, depending on the parameters of the optical system and the parameters of image processing of the device [31].

The obtained micrographs of the synthesized samples contained individual particles of irregular shape. The relative concentration of impurities c_{imp} was measured according to optical microscopy data:

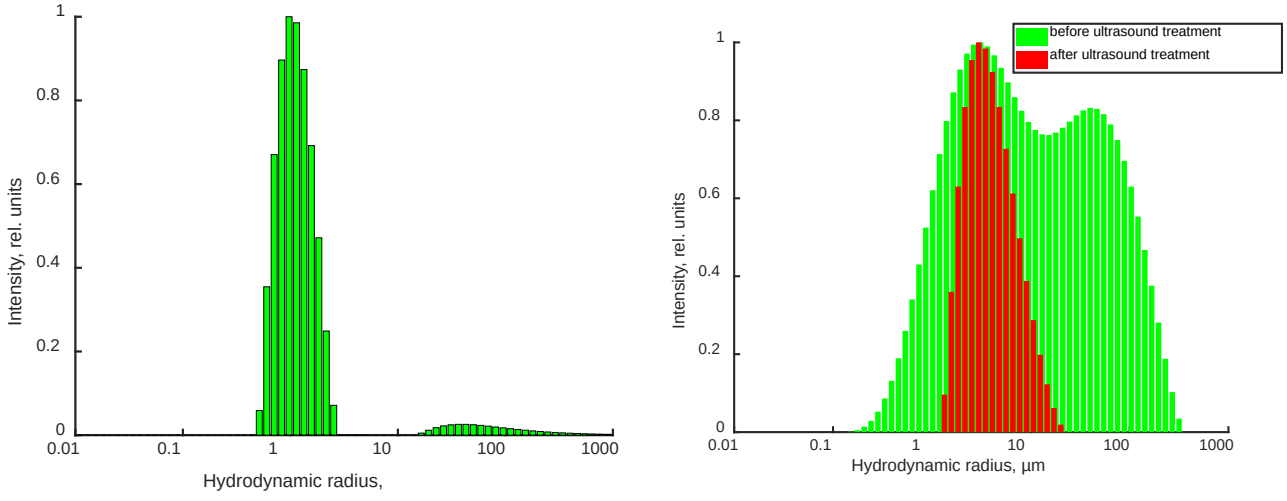
$$c_{imp} = \frac{N_{imp}}{N} \times 100\,\%$$

where N_{imp} is the amount of impurities, N is the total number of particles. Impurities included particles recognized in images with the coefficient of roundness less than 0.8 or with the diameter different from the average by more than 30%.

The measured values c_{imp} were 4.1% for sample No.1 and 8.4% for sample No.2. In this case, a significant part of the impurities were large particle aggregates. Since it is difficult to distinguish particle aggregates from particles closely located in microphotographs according to optical microscopy data, synthesized samples of liquid dispersions of microparticles were studied by the DLS method. The DLS method allows registering even small concentrations of large particles in a sample of a liquid dispersion.

As a result, it was found that the composition of the liquid dispersion of microspheres of sample No.2 contains particles of large sizes, while sample No.1 practically does not contain large aggregates of particles (Fig. 7).

Sample No.2 was sonicated in an ultrasonic bath with a frequency of 22 kHz and a power



a *b*

Fig. 7. The distribution of the scattered light intensity by particle size for samples No.1 (a) and No.2 (b)

of 75 W for 5 minutes. As a result of repeated measurements, it was found that there were no large aggregates in the dispersion (Fig. 7b). Thus, it can be concluded that the particles in sample No.2 are more prone to aggregation in comparison with sample No.1.

However, surfactants and stabilizers can be used for the prevention of the aggregation, moreover, particles used in immunofluorescence analysis have a shell of carboxyl groups, preventing particle aggregation [32, 33].

Thus, the revealed tendency to aggregation of particles in sample No. 2 should be taken into account in further study with microspheres. However, it is not a factor preventing the use of microspheres in immunoassay methods.

3.5. Magnetic properties

For the use of microspheres in planar immunoassay, they must have sufficient magnetic properties, for example, due to the high content of magnetite [34]. This condition is due to the need to immobilize the microspheres in the cell using a magnet for analysis [35].

In this study, the presence of magnetic properties in samples of synthesized microspheres was assessed visually using an optical microscope. A neodymium magnet was brought to the cell with microspheres in liquid dispersion. After that, the microspheres of both samples were aligned along the lines of magnetic induction, which indicates that the microspheres have magnetic properties.

The efficiency of immobilization of microspheres in a cell using a magnet was evaluated by analysing samples in a QuattroPlex device for multiplex immunofluorescence assay. During the analysis, the microspheres were immobilized in a flow cell using a magnet, after which fluorescence images were obtained in different fluorescence channels.

As a result of the study, it was found that the magnetic properties of the microspheres of both samples allow, firstly, to collect microspheres from the entire volume of the cell in one plane using a magnet, and secondly to keep them motionless for the time required to obtain fluorescent images in several channels (about 1 minute), preventing the displacement of microspheres under action of gravity, which is also important, since the displacement of microspheres in images obtained in different channels does not allow to take them into account when analysing images during multiplex immunoassay.

3.6. Fluorescence

Microspheres of each type were stained with Cy5 and Cy5.5 fluorescent dyes separately and with both dyes at different concentrations.

We studied such fluorescence parameters of synthesized microspheres as fluorescence intensity (brightness of microspheres in the image) and uniformity of dye distribution.

The brightness of the microspheres in the image was compared with the brightness of the reference particles. It should be noted that the reference particles contained other fluorescent dyes and the corresponding filters were used to obtain images. Otherwise, the experimental conditions were identical. The purpose of this experiment was to find out whether it is possible to obtain fluorescent images of samples of synthesized microspheres with a comparable exposure time in the camera, and also to draw conclusions about the required concentration of the dye.

Luminex microspheres with the highest and lowest dye concentration (regions 12 and 78) were used as reference.

The resulting 16-bit images contained a distribution of 2^{16} brightness gradations. The camera exposure was selected in a way, that the microspheres with the lowest dye concentration were distinguishable (that their brightness value in the image was twice the average brightness value for background noise), provided that the microspheres with the highest concentration of dye were not light-striking the image (i.e. their brightness value in the image did not exceed 65535 a.u.).

The images were analysed using the ImageJ software. Individual particles were identified by the intensity threshold in the image. Then the average intensity in the image of each detected particle was measured. In order to avoid false recognition of two aggregated particles as one, particles with a roundness value less than 0.8 were ignored. The roundness value was calculated using the formula (2).

As a result, for the synthesized samples, the selected exposure value in both fluorescence

channels (corresponding to Cy5 and Cy5.5) was 5 seconds for sample No.1 and 20 seconds for sample No.2, with a exposure time of 3.5 seconds for reference microspheres. Thus, we can conclude that for sample No.1 the selected dye concentrations were close to optimal, and for sample No.2 the concentration of the dye should be increased in order to reduce the analysis time.

The uniformity of the distribution of the dye for individual microspheres of the same sample is an important parameter, since it determines the spread of the brightness of the microspheres in the image. Due to the fact that the dynamic range of the camera is limited, the maximum possible number of distinguishable types of microspheres and, consequently, the multiplexing of the analysis depend on the spread in the brightness of one type of microspheres.

Based on the analysis of the obtained images, the values of the coefficient of variation of the fluorescence intensity were calculated, which characterize the variation of the brightness of microspheres of the same type (Table 1). In [36], a method for the synthesis of particles with a high degree of homogeneity and a fluorescence variation coefficient of 3.7%; was described, however, such particles had a large size (from 10 μm) and were not magnetic. Therefore, Luminex magnetic fluorescent microspheres were chosen as reference particles for comparison. The analysis was carried out on 5 fluorescence images for each sample of microspheres in both fluorescence channels.

It can be seen that microspheres of sample No.2 were characterized by a greater variation of brightness in both fluorescence channels. Thus, sample No.1 had the most uniform distribution of the fluorescent dye, which made it more promising for use in immunofluorescence analysis.

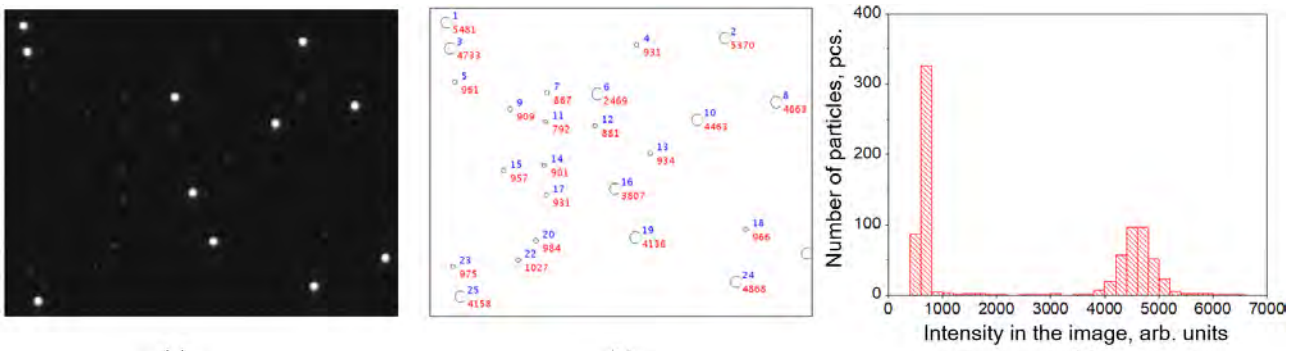
Fluorescence images for a mixture of two types of microspheres of sample No.1, differing in the concentration of one dye were also obtained. As a result, it was shown that it is possible to identify microspheres with different dye concentration by brightness in the image (Fig. 8).

Fluorescent images of two types of microspheres of sample No.1, containing both fluorescent dyes at different concentrations were also obtained.

As a result of the analysis of the obtained images, it was shown that the coefficient of variation of the fluorescence intensity in the

Table 1. Coefficient of variation of the fluorescence intensity of microspheres

Sample	Coefficient of variation of the fluorescence intensity, %	
	Cy5 / Dye 1	Cy5.5 / Dye 2
Sample No.1	8.8±0.2	9.4±0.3
Sample No.2	16.1±0.6	19.6±0.8
Reference microspheres	7.4±0.2	7.9±0.2



a *b* *c*

Fig. 8. a) Fragment of the obtained fluorescent image of a mixture of microspheres of sample No.1 with high and low concentration of the dye Cy5; b) analysis of the same fragment of the image: the upper number is the number of the particle detected in the image; the lower number is the average fluorescence intensity of the detected particle; c) the distribution of microspheres with different fluorescence intensities in the resulting image

image for both types of fluorescent dyes and for both samples of microspheres with different concentrations of dyes was also less than 10% (Fig. 9).

The low coefficient of variation of the fluorescence intensity allows encoding a large number of microsphere types, at least 36 regions, by introducing different concentrations of a fluorescent dye. This number is sufficient for the vast majority of applications of the multiplex immunofluorescence analysis.

4. Conclusions

As a result of the studies, it was found that the microspheres in both samples have optimal sizes for the use in immunofluorescence analysis and a high degree of monodispersity, do not contain a statistically significant amount of irregular impurities. The magnetic properties of the microspheres of both samples make it possible to quickly and reliably immobilize the microspheres in one plane of the measuring cell using a magnet. At the same time, the microspheres synthesized by the two-stage swelling method are characterized by a larger spread in the fluorescence brightness. Therefore, from the point of view of the uniformity of the distribution of the fluorescent dye, the use of microspheres synthesized by dispersion polymerization seems to be more promising for use in immunofluorescence analysis.

The possibility of identifying microspheres with different dyes and different concentration of dyes in the image was shown. The low coefficient of variation of the fluorescence intensity makes it possible to use a large number of types of spectrally encoded microspheres in immunofluorescence analysis, and, therefore, to identify a large number of pathogens simultaneously. The obtained results can be used to develop new diagnostic multiplex test systems based on spectrally encoded microspheres.

Conflict of interests

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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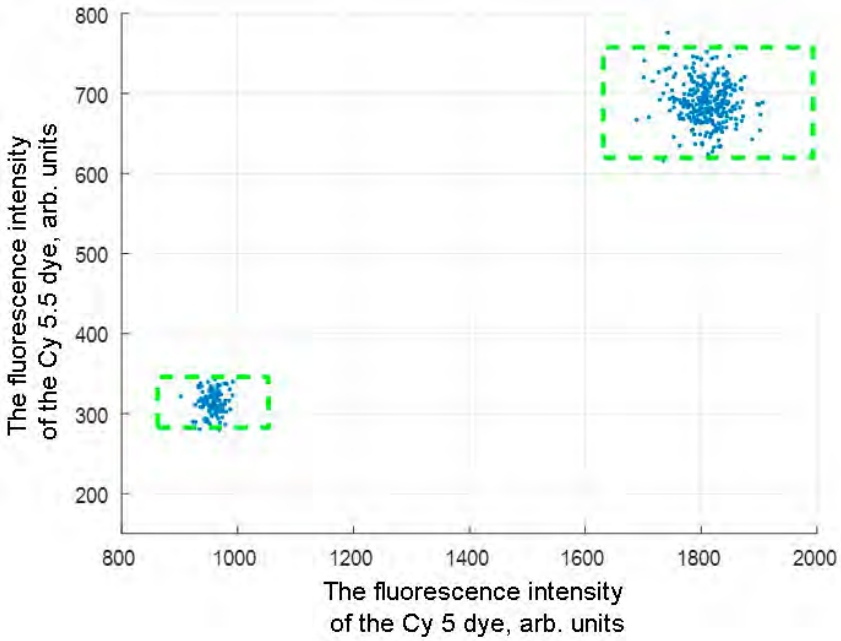

Fig. 9. The result of the analysis of the obtained fluorescent images of two types of microspheres with dyes Cy5 and Cy5.5 for the sample No.1. Dotted rectangles correspond to 10% deviation from the mean

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