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Use of Neural Network Algorithms for Elaboration of Fluorescent Biosensors on the Base of Nanoparticles

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Abstract—In this paper, the results of application of artificial neural networks for extraction of fluorescence contribution of nanoparticles used in biomedicine as biomarkers and drug carriers against the fluorescence background of inherent fluorophores of biological objects are presented. Principle possibility of solving this problem is shown. The used architectures of ANN allow detecting fluorescence of carbon dots against the background of proper fluorescence of egg protein with reasonably high accuracy—not worse than 0.002 mg/mL.

Keywords: fluorescence, carbon dots, biomarkers, autofluorescence, artificial neural networks, data aggregation

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INTRODUCTION

At present time, the task of elaboration of new photostable fluorescent biosensors being able to replace dye molecules, which are usually used in biomedicine, is very urgent [1–3]. Molecules of organic dyes have high brightness of luminescence per unit mass but these fluorescent sensors have limited capabilities for long-term in vitro and in vivo control because of effect of rapid photobleaching and cellular toxicity. In order to overcome these shortcomings, fluorescing nanoparticles – quantum dots and nanodiamonds – were suggested as alternative to dye molecules.

Quantum dots (QD) and nanodiamonds (ND) are new perspective materials for optical biovisualization because of their exclusive photostability at room temperature and high quantum efficiency [4–8]. Besides fluorescent ability, QD and ND have multi-functional surface which can be modified according to the problems formulated. By different functionalization of the surface of nanoparticles, one can enhance their biocompatibility and reduce their toxicity [8–10] in living cells.

One of the most important problems of this area is elaboration of methods of visualization of nanoparticles in biological material. Now the most widespread method of study of biological processes is visualization by means of fluorescence. A serious problem in such studies is background fluorescence caused by fluorescence of inherent fluorophores in biological tissue. Most important tissue fluorophores are tryptophan, phenylalanine, tyrosine, collagen, flavins and flavoproteins, beta-carotene, porphyrins, nucleic acids, coenzymes, vitamins, pigments etc. Spectrum of tissue autofluorescence is a result of superposition of fluorescence bands of many tissue fluorophores. Such autofluorescence appreciably impedes observation over existent processes and movements of fluorescing nanoparticles. That is why elaboration of method of extraction of fluorescence signal of nanoparticles-markers against the background of proper fluorescence of biological tissue and control of its change for providing biomarker tracking are very urgent.

Now problem of background fluorescence is solved mainly by two ways: (1) in the area of material science—by searching for optimal selection of properties of nanoparticles and for method of modification of their surface [9–11]; (2) in the area of experimental equipment – to reduce background signal, laser exciting radiation is focused in a very small volume which necessitates considerable increase of price of devices [12].

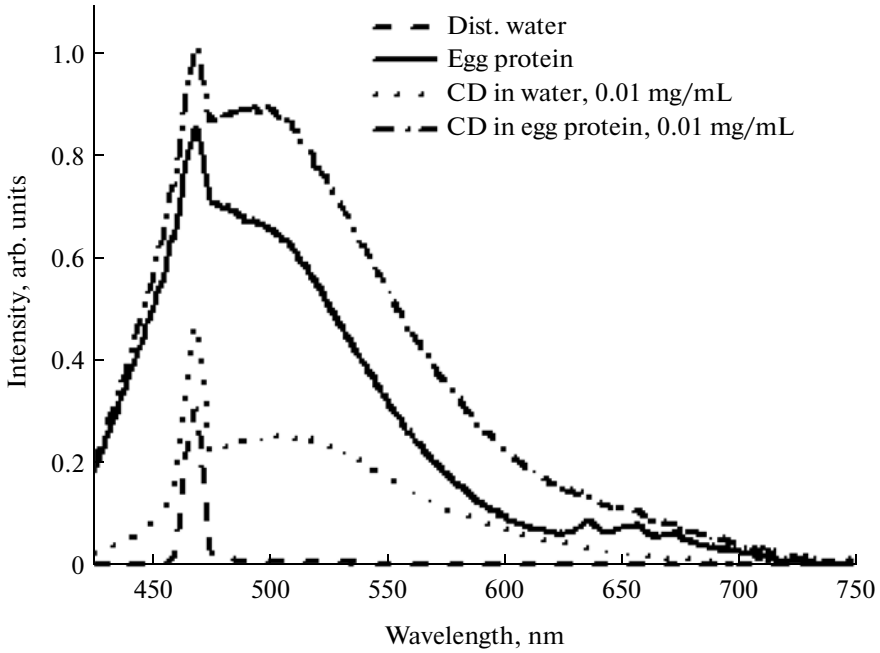

Fig. 1. Spectra of RS and FL of distilled water, water suspension of CD (0.01 mg/mL), egg protein and egg protein with introduced nanoparticles (concentration of CD in protein 0.01 mg/mL).

In this paper it is suggested to solve the problem of extraction of fluorescence of nanoparticles against the background of inherent fluorescence of biomaterial by method of pattern recognition—using artificial neural networks.

In the papers of Russian and foreign researchers, a large number of methods and models which can be used for detection and recognition of objects by their images, were suggested [13–15]. Distinctive feature of use of such methods for solution of concrete practical problems is the fact that solution of every of such problem requires specific additional studies and developments. That is why along with use of known methods in their classical form, active studies in the area of further increase of accuracy and efficiency of neural network solution of different inverse problems are continued [15–18]. In spite of very extensive use of methods of pattern recognition in biomedicine, papers about application of these methods to the problem of extraction of fluorescence of nanoparticles against the background of inherent fluorescence of biological tissue are not known to the authors of this paper.

The aim of this study is elaboration of methods for application of neural network algorithms for extraction of optical response of a certain component against the background of overlapping optical responses of other components in a complex multi-component system.

OBJECTS OF RESEARCH

It is known that nanoparticles produced as a result of reactions of oxidizing of carbon materials have fluorescent properties; they are biocompatible, non-toxic and can be used as fluorescent biosensors [19–23]. In this paper, biosensors were elaborated on the base of quantum carbon dots (CD) which were synthesized in the International Technology Center in USA (North Carolina) [21–22].

As biological material, protein from hen's egg was used. Advantage of choice of this biomaterial lies in simplification of introduction of nanoparticles in the cell (egg is a single cell). It is known that introduction of nanoparticles and control of their presence inside the cells is a special experimental problem.

Figure 1 presents spectra of Raman scattering (RS) and fluorescence (FL) of distilled water, CD water suspension (0.01 mg/mL), egg protein and egg protein with introduced nanoparticles (concentration of CD in protein is 0.01 mg/mL). Optical signal excitation wavelength is 405 nm. Spectrum of inherent FL of egg protein in the studied spectral area consists of several bands: intense broad unstructured band from 430 to 730 nm with maximum near 480 nm and weaker bands with maxima near 640 nm, 655–660 nm, 675 nm.

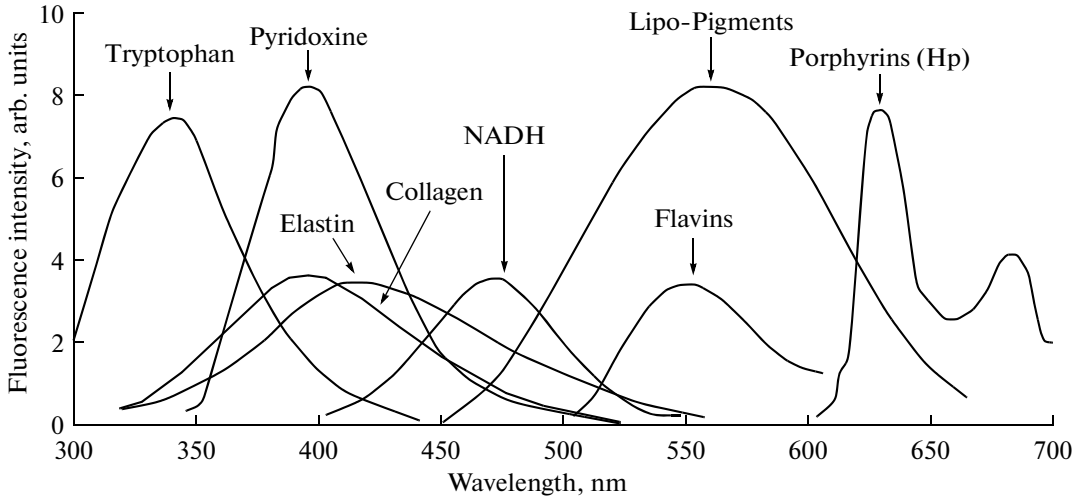

Fig. 2. Spectra of fluorescence of inherent fluorophores of biological tissues [24].

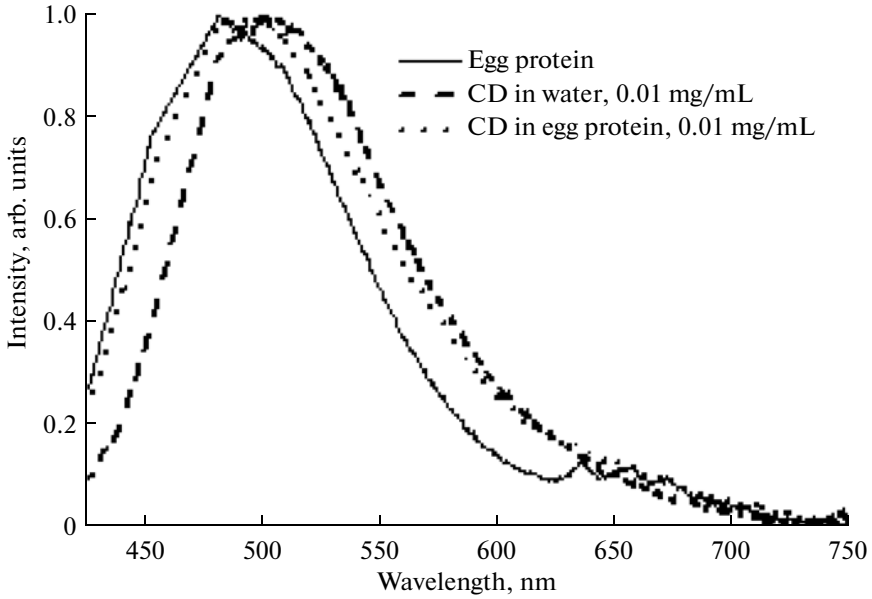

Fig. 3. Spectra of fluorescence of egg protein, CD in water and egg protein with introduced CD. Spectra were normalized to their own maximal intensity.

As it is known, inherent fluorescence of biological tissues is provided by such fluorophores as tryptophan, phenylalanine, tyrosine, collagen and elastin; flavins and flavoproteins, beta-carotene, porphyrins, nucleic acids, coenzymes etc. [24]. Tissue autofluorescence spectrum is a result of overlapping of FL bands of a great number of such fluorophores and it spreads from 250 to 700 nm (Fig. 2).

From Fig. 1 and Fig. 2 it follows that the main band of egg protein fluorescence is formed by fluorescent contributions of collagen, elastin, pyridoxine, NADF, flavins, lipo-pigments. Weak FL bands near 640–670 nm are caused by fluorescence of porphyrins. CD fluorescence lies from 430 to 680 nm with the maximum near 500–505 nm (Fig. 1). So spectra of fluorescence of CD and egg protein substantially overlap but differ by position of maxima (Fig. 3). Spectrum of FL of egg protein with introduced CD at the specified concentration of CD in protein also represents broad unstructured band from 400 to 730 nm with maximum near 490–495 nm (Fig. 3).

Obviously, under change of CD concentration in protein, the band of their combined FL will be considerably changed because of several reasons. Main of these reasons are: (1) under change of CD concen-

tration, the intensity of CD fluorescence changes; (2) due to various interactions of CD with protein components, fluorescence of both protein and CD changes. At that, these interactions are very complex, and they are still far from being studied, partly because carbon nanoparticles were synthesized relatively not long ago. Because of these reasons it is impossible to elaborate a model of change of band of combined FL of egg protein and CD under change of CD concentration (for example, when CD moves in biotissue) by traditional mathematical methods. It means that the direct problem cannot be solved by traditional mathematical methods, and therefore the inverse problem of extraction of fluorescence contribution of a varying quantity of CD against the background of protein FL cannot be solved, too.

In this paper, solution of the problem of extraction of fluorescent contribution of CD against the background of inherent protein FL was performed by algorithms of artificial neural networks (ANN).

METHODS AND APPROACHES

In the context of solution of the specified inverse problem of optical biopsy – extraction of fluorescent contribution of nanoparticles against the background of autofluorescence of biological tissue using ANN—the following methods are being elaborated by the authors of this paper:

(1) Method of detection of CD fluorescence against the background of autofluorescence of biotissue by total fluorescence spectrum of the sample.

The considered problem is the simplest variant of classification problem—determination of the belonging of a pattern to one of two non-crossing classes (nanoparticles present—no nanoparticles). In solution of this classification problem, it is necessary to take into account specifics of input data—there are no individual features allowing one to ascertain confidently belonging of the pattern, i.e. it is not a problem of classical spectral analysis. On the contrary, spectra of fluorescence of nanoparticles and biofluorophores almost completely overlap, although they have different shape. This peculiarity causes expediency of application of neural network methods for solution of the stated problem and requires elaboration of correct methodology of its solution. This methodology includes elaboration of procedure of data preparation, determination of optimal neural network architectures, algorithms and parameters of their training.

Solution of the problem of detection of nanoparticles by their fluorescence in biological tissues will allow monitoring the track of biomarkers and making sure of targeted delivery of biologically active compound attached to the nanoparticle to the required place.

(2) Method of determination of minimal CD concentration when presence of nanoparticles is confidently detected against the background of proper fluorescence of biotissue.

In fact this means determination of the threshold of sensitivity of the method as a whole. It is clear that the numeric value of this threshold depends both on methodology of experiment and on further data handling.

(3) Method of solution of the inverse problem of determination of relative concentration of nanoparticles in biomaterial.

The considered inverse problem is rather complicated, but without its solution the problem of drug delivery by fluorescing nanoparticles remains unsettled. To estimate the quantity of drugs or biologically active supplements delivered to the target receptors, it is necessary to determine concentration of nanoparticles that achieved their targets. Herein, taking into account the specifics of initial data is also required. However, the methodology of solution of this problem will differ from methodology of solution of the detection problem: the criterion of decision-making in selection of one or another algorithm will be the error of inverse problem solution using this algorithm.

(4) Use and comparison of algorithms of input data compression.

One of the traditional problems arising in work with spectroscopic data is very high dimensionality of the input data, as a spectrum is usually registered in several hundred or even thousand channels. At the same time, the number of patterns for ANN training when using the "experiment-based" approach (ANN training using only experimental data) corresponds to the number of measured spectra, and therefore it is substantially limited [25]. Both of these factors reduce the ratio of the number of patterns and the number of input features. This is unfavorable for ANN training. Therefore, it is necessary to elaborate a method to reduce the dimensionality of the input data.

Use of ANN for solution of inverse problems of optical spectroscopy is possible in the context of three approaches: "model-based", "experiment-based", "quasi-model" [25–27].

In the "model-based" approach, to obtain the data for ANN training, an existing analytical or computational model of solution of the direct problem is used. If such model exists, it is possible to provide the necessary representativity of all the data sets essential for ANN training. However, the quality of the solution in this case directly depends on adequacy of the used model. In the situations when creation of an adequate model is impossible due to complexity of characterization of the object, this approach is unusable.

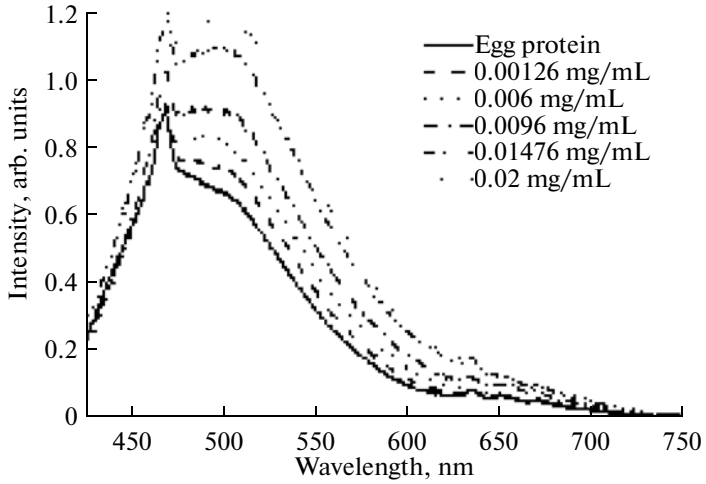

Fig. 4. Spectra of RS and FL of initial egg protein and egg protein with introduced CD with different concentrations.

In the "experiment-based" approach, experimental data are used for ANN training. The shortcoming of this approach is insufficient representativity of the data sets, since obtaining of immense experimental material is a reasonably tedious work. The main advantages of this approach are: when ANN trains directly on experimental data, all molecular interactions are taken into account; the network is trained with real instrumental noise which raises accuracy of solution of inverse problems.

In the "quasi-model" approach, model spectra are used to obtain representative data sets. Unlike the "model-based" approach, where data are calculated according to known analytical expression for description of spectra, in the "quasi-model" approach first a parametrical "quasi-model" for description of spectra is constructed on the base of a moderate set of experimental data, and then it is used to calculate the complete data array. It is obvious that in such a way one can obtain enough patterns and provide good representativity of data sets for ANN training, unlike the "experiment-based" approach. However, the accuracy of solution of the inverse problem in the "quasi-model" approach heavily depends on two factors: (1) on inaccuracy of the "quasi-model" used for calculation (i.e. on how well does the chosen or constructed calculation quasi-model correspond to reality); (2) on difference between noise in the calculated patterns and real noise in experimental data.

As it was mentioned above, in the considered problem of recognition of fluorescent contribution of CD against the background of fluorescence of egg protein, the "model-based" approach cannot be used because of lack of correct analytical description of fluorescence spectra of CD and protein.

In this study, the inverse problem was solved using ANN in the context of "experiment-based" approach. Since the object of research is living biological material whose condition can appreciably change as a result of long-term laser irradiation, it is difficult to get a representative set of experimental data. That is why it was impossible to implement the "experiment-based" approach with maximal efficiency.

EXPERIMENT

Spectra of egg protein with introduced CD were obtained using a laser spectrometer. RS and FL were excited by a diode laser (wavelength 405 nm, incident power on the sample 50 mW). Spectra were measured in step-by-step mode with registration by PMT in the range 430–750 nm. Spectral resolution was 0.5 nm. Temperature of samples during experiment was stabilized at 22.0 ± 0.1°C. Spectra were corrected to laser power and accumulation time. Further mathematical processing of spectra consisted in subtraction of pedestal caused by elastic scattering of light in the cuvette with the sample, and normalization of spectra to the area of water Raman valence band.

Two series of spectra of RS and FL were obtained in the experiment for two different egg proteins with introduced CD in the concentration range from 0 to 0.02 mg/mL with increment 0.00075 mg/mL. In Fig. 4, one can see some experimental RS and FL spectra of egg protein with CD at different concentrations. The obtained data array was used to solve the stated inverse problem using ANN.

Table 1. Values of the coefficient of multiple determination R squared on various data sets for various algorithms of data processing

Algorithm \ Data set	Training	Test	Series 1
Perceptron	0.995	0.887	0.500
GRNN, USF	1.000	0.893	**0.883**
GRNN, ICSF	1.000	0.960	**0.878**
GMDH	0.981	0.982	–0.030

USE OF ANN. RESULTS AND DISCUSSION

To implement the "experiment-based" approach, both available series of experimental spectra were used: Series 1 (15 spectra in the CD concentration range from 0 to 0.02 mg/mL) and Series 2 (28 spectra in the same concentration range). All spectra in a series were obtained for the same protein, but different series were obtained for different proteins. That is why it was decided to train ANN using Series 2, and Series 1 was used as the set of independent data for examination and to test stability against change of protein.

For correct realization of methodology of ANN training, it is necessary to divide the whole data into three data sets: training, test and examination. The training set is used directly for ANN training (adjustment of weights); the test set—for periodic testing during process of training in order to determine the moment when training must be stopped to avoid ANN overtraining; the examination set – to test the quality of ANN operation on independent data. In this study, 28 patterns of training data (Series 2) were divided in the following way: 23 patterns to the training set, 5 patterns to the test set. As the number of patterns was very small, the division was performed in a regular manner (every 5-th pattern in the order of increasing concentration of CD was taken to the test set). The data of Series 1 were used as the examination set. So operation of the obtained networks was assessed not just by independent data from the same experiment, but by data from another experiment. This provided estimation of stability of the solution against change of object and experimental conditions.

The following adaptive algorithms were used to work with this problem: (1) Perceptron with one hidden layer, trained by the algorithm of error backpropagation [28]; (2) General regression neural network [29]; (3) Group method of data handling [30]. For all the calculations, software package NeuroShell 2 [31] was used.

Because of very small number of patterns, use of perceptrons with several hidden layers was not appropriate. Prior studies demonstrated that perceptron with 10 neurons in the single hidden layer was optimal for this problem. However, influence of the number of neurons in the hidden layer is not so important when using a test set for determination of the moment for termination of training.

In this study, the following parameters of perceptron were used: transfer function in hidden and output layers—hyperbolic tangent, learning rate—0.01, moment—0.5, pattern presentation order – random, stop training criterion—1000 epochs (23000 events) after minimal error on test set.

For General Regression Neural Network (GRNN), two variants were considered: with unified smoothing factor (USF) selected by step-by-step method, and with individual corrections to the smoothing factor (ICSF) for every input feature. In the second case, the values of the corrections, as well as the value of the main smoothing factor, were selected by genetic algorithm [31]. In both cases, selection was performed by minimal squared error on test set.

Group Method of Data Handling (GMDH) has the advantage that it constructs a regression analytical model with optimal complexity in the given basis of support functions (in this case, polynomial basis was used), and the obtained model due to the special procedure of its construction rarely turns out to be overtrained. However, it may not possess sufficient stability in respect to change of some parameters of the problem or of experimental conditions. So in this case one could expect that GMDH model would show good results on data sets from the initial array of training data, but could show low results on data from Series 1 obtained using slightly discrepant experimental material (protein of another egg).

In Table 1 and Table 2, the results obtained with the four described adaptive methods on three data sets (training, test, examination – Series 1) are presented. The results obtained on examination set are most informative. As statistical indexes, the coefficient of multiple determination R squared (Table 1) and the mean absolute error of measurement of CD concentration (Table 2) are adduced.

The obtained results allow us to make the following conclusions.

Table 2. Values of the mean absolute error (MAE) in mg/mL on various data sets for various algorithms of data processing

Algorithm \ Data set	Training	Test	Series 1
Perceptron	0.00034	0.00154	0.00405
GRNN, USF	0.00000	0.00164	**0.00172**
GRNN, ICSF	0.00000	0.00066	**0.00176**
GMDH	0.00064	0.00061	0.00584

Table 3. Values of the coefficient of multiple determination R squared on various data sets for different degrees of aggregation

Degree \ Data set	Features	Training	Test	Series 1
Perceptron				
Initial	651	0.995	0.887	0.500
A32	20	0.972	0.990	0.673
A65	10	0.874	0.906	0.854
A93	7	0.875	0.894	**0.877**
GRNN with unified smoothing factor				
Initial	651	1.000	0.893	0.883
A32	20	0.999	0.905	0.890
A65	10	1.000	0.904	**0.896**
A93	7	0.997	0.908	0.891

(1) Best results on examination set were demonstrated by both modifications of GRNN. Perceptron showed comparable results on training and test sets but turned out to be substantially less stable against change of experimental conditions. This is demonstrated by the results obtained on examination set (Series 1).

(2) As it was expected, the worst stability was demonstrated by GMDH and it is not surprising. With such a small number of patterns in the training array (28), the method can construct only very simple models showing sufficiently high results on the training array, but incapable of data generalization.

(3) For both modifications of GRNN, mean absolute error on examination set (for Series 1) was about 0.0017 mg/mL (Table 2). This makes it possible to state that minimal detectable concentration of CD against the background of fluorescence of biotissue does not exceed 0.002 mg/mL.

The considered problem in its initial statement, as it was repeatedly mentioned above, is characterized by extremely unfavorable ratio of the number of patterns in training set (23) and the number of input features (651). That is why the next direction of studies was consideration of algorithms of reduction of the input dimensionality of the problem, i.e. reduction of the number of input features.

Previous investigations of the authors of this study demonstrated [32] that one of the most efficient ways to reduce dimensionality of spectroscopic data is aggregation of channels, when new composed features constitute sums of intensities in several adjacent spectral channels. Apart from possible improvement of the quality of solution of the problem, this approach in case of success can allow using considerably less expensive equipment with significantly lower spectral resolution.

In Table 3 and Table 4, one can find the results obtained by perceptron and by GRNN with unified smoothing factor on the three data sets (training, test, examination) for different degrees of uniform aggregation (by 32, 65 and 93 channels). Herewith the number of input features decreased from initial 651 to 20, 10 and 7 features, respectively.

Here the results obtained on examination set are most informative, too. As statistical indexes, the coefficient of multiple determination R squared (Table 3) and the mean absolute error of measurement of CD concentration (Table 4) are adduced.

Table 4. Values of the mean absolute error (MAE) in mg/mL on various data sets for different degrees of aggregation

Degree \ Data set	Features	Training	Test	Series 1
Perceptron				
Initial	651	0.00034	0.00154	0.00405
A32	20	0.00088	0.00045	0.00302
A65	10	0.00183	0.00119	0.00181
A93	7	0.00183	0.00129	**0.00165**
GRNN with unified smoothing factor				
Initial	651	0.00000	0.00164	0.00172
A32	20	0.00010	0.00141	0.00172
A65	10	0.00007	0.00143	**0.00170**
A93	7	0.00018	0.00135	0.00170

The presented results allow making the following conclusions.

(1) Aggregation of channels allowed significantly improving the results for perceptron. Note that the quality of solution of the inverse problem of determination of CD concentration monotonously improves with intensification of compression, i.e. with decreasing number of input features. The best result was achieved with 7 input features.

(2) For GRNN which is not so sensitive to the ratio of the number of patterns and the number of input features, the dependence of the result on the number of input features is not so evident. The best result is achieved with 10 input features. At that, both neural network architectures show comparable results.

(3) The minimal obtained value of the mean absolute error of determination of CD concentration on examination data set, which corresponds to minimal detectivity of the method, was 0.00165 mg/mL. Because of lack of patterns, this result can depend on which patterns exactly are selected for test data set. However, such dependence cannot significantly influence the result. Anyhow, one can assert that the used ANN architectures allow detection of fluorescence of carbon dots against the background of inherent fluorescence of egg protein with accuracy not worse than 0.002 mg/mL.

CONCLUSIONS

In this paper, principle opportunity of solution of the inverse problem of optical visualization—extraction of fluorescence of nanoparticles against the background of inherent fluorescence of biological environment using neural network algorithms has been demonstrated. It has been shown that the methods used allow detection of fluorescence of CD against the background of inherent fluorescence of egg protein with sufficiently low concentration threshold of detection (not greater than 0.002 mg/mL). Note for comparison that to obtain contrasting image of fluorescence of nanoparticles in living cells by confocal optical microscopy, the operating concentration of water suspension introduced into the cell equals 1 mg/mL.

It has been also demonstrated that use of input data compression by aggregation of initial spectral channels allows additionally increasing the quality of solution of the inverse problem of extraction of CD fluorescence against the inherent background fluorescence of biological object.

As the direction of further research, one should give consideration to using "quasi-model" approach, in which it will be possible to get a greater number of patterns for training and test data sets.

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