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# Influence of polyacrylic acid nanoparticles on the elastic properties of RBCs membranes in patients with diabetes mellitus type 2

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**Abstract**

The influence of polyacrylic acid (PAA) derived nanoparticles, a star shaped macromolecules with hydrodynamic radius of 14 nm, on the structure and mechanical properties of red blood cells (RBCs) membranes in patients with diabetes mellitus type 2 by AFM method has been studied. Their behavior has been compared to that of linear PAAs with different degrees of polymerization. Significant changes in the structure of RBCs membranes did not occur and traces of polymer chains or nanoparticles were not found. It was shown, that changes of the elasticity modulus were observed after cells incubated in the presence of linear PAA with $M_n$ =20000 Da and 225000 Da, due to the interaction of polymer chains with the components of the cell membrane. The star shaped NPs with $M_n$ = 57000 Da lead to the smallest changes in the structure and properties cells membrane. Thus, these NPs can be recommended for drug delivery applications.

**Keywords:** Red blood cells, nanoparticles, poly(acrylic acid), elasticity modulus, atomic force microscopy

## 1. Introduction

Diabetes mellitus type 2 (DM2) one is good studied illness with proven treatment regimen. Often DM2 is combined with other pathologies. As a rule, DM2 accompanies dyslipoproteinemia, and as a result patients have a number of complications of the cardio - vascular system such as hypertension, coronary heart disease. In addition, the long-term compensation creates unsatisfactory results in the development of diabetes, including micro- and macrovascular complications, subsequently causing adverse outcomes.

As a result of DM2 changes in structure and mechanical properties of erythrocytes and platelets membranes, there is a dysfunction of blood microcirculation. Not enough attention has been focused on aggregation of RBCs, which plays one of the important roles in the blood microcirculation. The effect of oxygen transport in the microvessels depends on RBCs aggregation and deformability in significant degree.

Till now there are no drugs which can correct this pathology properly. One of the important directions in modern nanoscience is the application nanoparticles (NP) as containers for local drug delivery, therefore studies on their influence on cell membranes are also necessary. NPs have wide range of applications in biotechnology, biomedicine and pharmaceutical sciences. NPs, such as gold [1], silver [2], solid lipid NPs [3] and nanowires [4], titanium dioxide [5], magnetite [6], iron oxide [7], as well as polymer derived NPs [8] have been currently used for local drug delivery. They display a number of demerits, such as stimulation inflammatory disease, demand additional influence of radiation that not always is favorable for the functional properties of the cells. Polymeric NPs can be prepared through

simple methods, but it is difficult to create uniform structure and small size. As shown in the article [9] polystyrene (PS) NPs modified with carboxyl groups (220, 450, 830 and 110 nm) adhered on to RBC membranes. Erythrocyte-bound NPs did not adversely affect the circulation of the erythrocytes themselves. Although the particles were eventually cleared from the circulation, the cells had normal circulation lifetimes. So, this PS NPs can be used as drug delivery carriers which do not break functional RBCs properties and microcirculation blood process. These promising results open the perspective for further investigations on macromolecular species or nanosized particles bearing carboxylic functions.

In recent years atomic force microscopy (AFM) method has been widely uses for estimation the influence of different NPs on the blood cells membranes elastic properties. This method provides information and visualization of surface structure and properties on nano- and microlevel. The information about the structural properties of red blood cells concerns important indicators for determining the healthy, diseased or aging status of erythrocytes. In this aspect, AFM can be used as disease diagnostic tool.

The aim of the present work is to estimate the influence of polyacrylic acid spherical NPs and linear chains on the elastic and adhesion properties of the erythrocytes membranes in patients with diabetes mellitus type 2 using AFM technique.

## 2. Methods

The influence of the linear and star-shaped polyacrylic acids on the structure and mechanical properties of red blood cells (RBCs) membranes was estimated in two groups of patients: the first group included patients with diabetes mellitus type 2 and diabetic nephropathy (n=15; 54,3 $\pm$ 4,9 yrs), the second group - patients with diabetes mellitus type 2, diabetic nephropathy and arterial hypertension (n=20; 55,6 $\pm$ 5,2 yrs). All patients had different grades of hyperlipidemia. Concentrations of glycated hemoglobin (HbA1c), total cholesterol (TC) and triglycerides (TG) were determined by described below methods.

Blood sampling was carried out from the cubital vein no earlier than 12 hours after the last meal. Biochemical blood tests were carried out in the morning no later than 2 hours after blood collection. The content of TC, TG plasma levels were measured by enzymatic assay using reagents and equipment of «CORMAY» company. The level of HbA1c was determined by reference method - liquid ion exchange chromatography high pressure on an automated analyzer D10. The variation coefficient is less than 4% (1-1.5% in practice).

Before the preparation of samples for AFM measurements we studied articles in which these methods were described. As rule, researchers used ethanol and glutaraldehyde (GA) with different concentration [10], chloroform [11] or fixing cells in the air, and then doing smear on the glass or mica plates. It was shown, that the best method for AFM research is fixing in the air for estimation of the membrane structure. But more often RBCs are fixed with using GA, because such preparation doesn't cause oxidation process and membranes properties remain unchanged.

Erythrocytes were separated from whole blood, stabilized with potassium ethylendiaminetetraacetatic acid dipotassium salt ($K_2$EDTA) (Sigma-Aldrich), centrifugated for 3 min at 90g. Then the precipitate was washed with phosphate buffer 3 times and added solution of the corresponding polyacrylate. Poly(acrylic acid)s (PAA) with linear chains (average molecular weights $M_n$= 6000, 20000, and 225000 Da) and NPs with architecture of star-shaped macromolecules in physiological solution (c = 0.2 mg/ml) were used to effect the RBCs suspension. The suspensions were incubated at room temperature for 20, 40 and 60 minutes, then washed from the macromolecular species and fixed with glutaraldehyde (Sigma-Aldrich) on the plates of mica. Fixation was carried out by adding 700 μl 0.5% solution of GA. It was mixed and left for 30 minutes. Then solution was centrifugated for 3 min at 90 g. Liquid supernatant was removed. At the next step 700 μl phosphate buffer was added and centrifuged for 3 min at 90 g two times. Then supernatant was removed and 700 μl distilled water were added, centrifugated for 3 min at 90 g twice. At the last step we gathered half of the supernatant. The precipitate and residuary supernatant was mixed and the content from test-tube was smeared as monolayer on mica plate. Samples were dried for 2 h.

Red blood cell aggregation was assessed by measuring the erythrocyte sedimentation rate (ESR) in capillaries with height 200 mm and diameter 3 mm [12]. Structure and mechanical properties of the RBCs membranes were estimated with using standard silicon cantilevers (Mikromash, K= 3 N/m, R= 30 nm). Elasticity modulus was calculated by Hertz model, adhesion force – by Jonson-Kendall-Robertz equation. Change of properties of the RBCs membranes were analyzed for two groups of patients: men and women.

The synthesis of the star polymer was described previously [13]. The linear poly(acrylic acid) with $M_n$=6000 Da and $M_w/M_n$=1.03 was synthesized through atom transfer radical polymerization applying procedure similar to the described one in [13] for the synthesis of the acrylic arms, while the polymers with average molecular weights $M_n$=225000 and 20000 Da were commercial products (Polyscience, Inc). Dynamic light scattering (DLS) measurements were performed at 25 $^oC$ on a Brookhaven BI-200 goniometer with vertically polarized incident light of wavelength $\lambda$ = 632.8 nm supplied by a He-Ne laser operating at 35 mW and a Brookhaven BI-9000 AT digital autocorrelator. Measurements of the scattered light from the aqueous solutions were made at angle 90$^o$ to the incident beam.

## 3. Results and Discussion

The polymer species used in the present study were: (i) a core–shell type star polymer whose interior forms hyperbranched polystyrene bearing arms of poly(acrylic acid) with molecular weight $M_n$=57 000 Da and (ii) linear polyacrylic chains with average molecular weights $M_n$=6000, 20000 and 225000 Da. The star macromolecules possessed in average ten arms with degree of polymerization DP=58. The shape of macromolecules is spherical with hydrodynamic radius $R_h$=14 nm determined by diffusion ordered NMR spectroscopy [13] and monomodal size distribution (Fig. 1). DLS measurement gave a lower value for the apparent hydrodynamic radius of the NPs because of the highly swollen shell of the construct which cannot effectively scatter the light. On the other hand, the linear poly(acrylic acid) chains adopt rod like shape under physiological conditions.

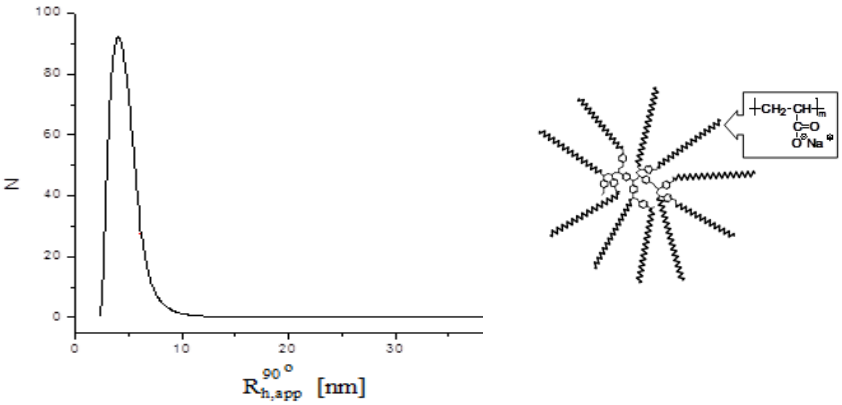



Fig. 1. The size distribution of the star polymer NPs in aqueous solution at pH 7 and 25 °C (left graph); schematic presentation of the star-shaped macromolecules (right scheme).

The structure of erythrocytes membranes before and after exposition with linear PAAs was studied. It was shown, that mean square roughness ($R_q$) was changed after incubation for a period of 40 min and decreased from 12 nm to 9 nm. More long influence causes to increase $R_q$ to 14.2 nm. Roughness is defined on a scan region 1 μm to 1 μm. These changes are due to alteration in membrane properties on nanolevel after incubation with the polymer species. The example of RBCs membranes structure changes is shown on Fig. 2 at the Torsion regime of AFM. Traces of polyacrylates on the surface of RBCs were not found.

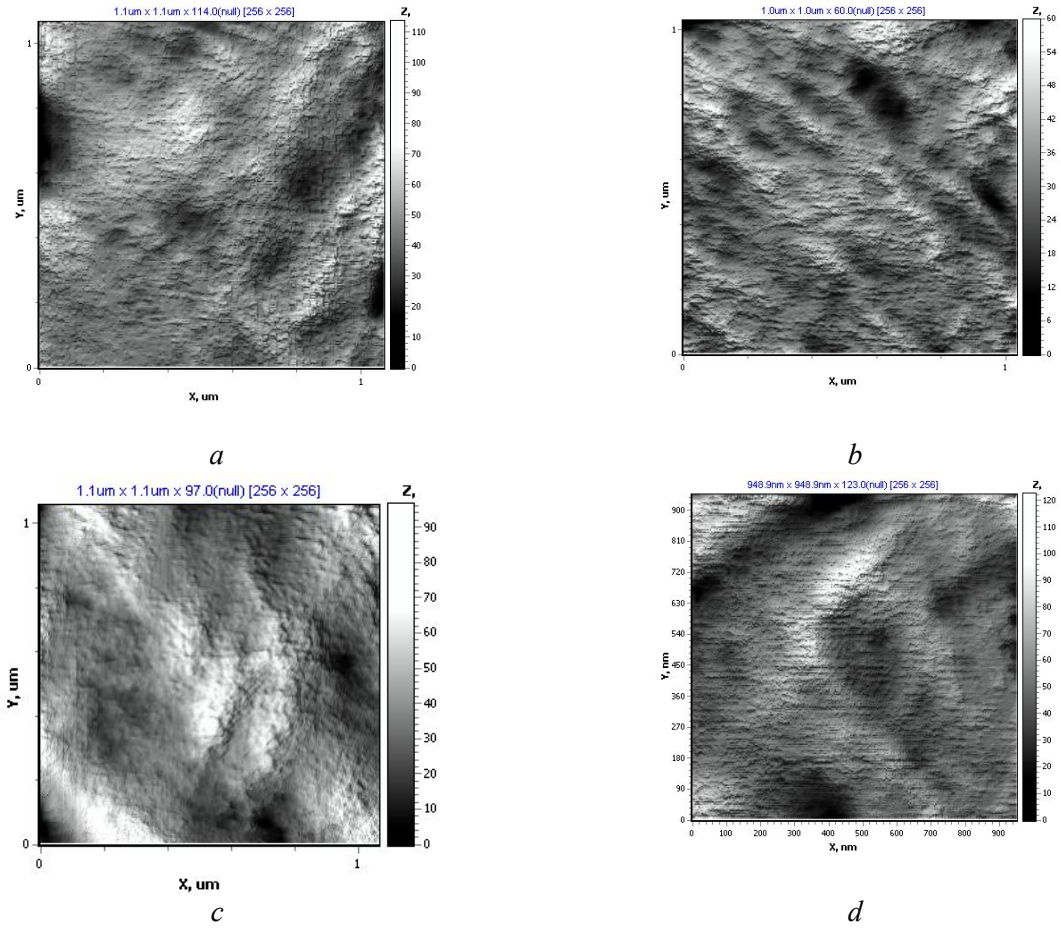



Fig. 2. Structure of erythrocytes membranes before (a) and after different time of incubation with PAA NPs (star-shaped) (b- 20 min; c – 40 min, d – 60 min), area 1x1 μm, Torsion regime

Previously, it was shown that elasticity modulus of the membrane in healthy subjects is lower than in diabetes patients [14]. In the present study, we compared the values of the modulus in the two groups of patients with DM2, described above. RBCs membranes elasticity modulus of men in the first group was 50% lower than analogous values in the second group. Probably, it was due to higher TG level in patients of the second group. Significant differences of the elasticity modulus rates in the second group between men and women were not found.

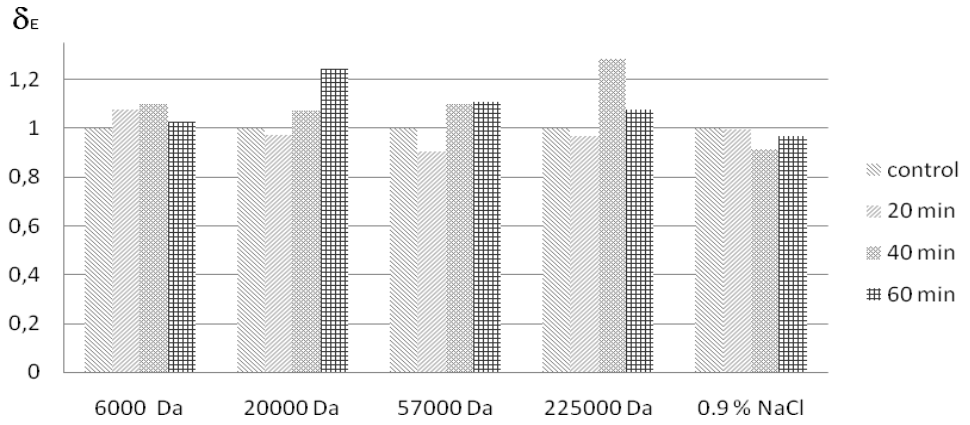
The influence of the PAA NPs is illustrated on Fig. 3. On the graphic the values of the relative modulus are presented ($\delta_E$). $\delta_E$ was calculated relative to the value of the elasticity modulus of the control samples.

(1) $$\delta_E = E/E_{contr}$$

$E$ - elasticity modulus of RBCs membranes sample after incubation with NPs;
$E_{contr}$ - elasticity modulus of RBCs membranes control sample

It was shown, that mechanical properties were not changed after treatment with PAA with $M_n$=6000 Da and star macromolecules with 57000 Da and 0.9% NaCl on RBCs membranes of patients in both groups (modulus was changed less than 20%). A small percentage of modulus change was characterized after influence NP with $M_n$ =20000 Da in two groups of patients and NP with $M_n$ =225000 Da on RBCs of men and women in two groups of patient. But significant changes (about 50 %) were established for men in the second group after cells' incubation with NP of PAA $M_n$ =20000 Da

during 40 min. Similar changes were established for men in the first group after RBCs incubation with PAA NP, $M_n$ =225000 Da during 40 min. Physiological solution causes no changes of the mechanical properties of RBCs membranes. These changes linked to structure PAA NP and interaction with components of RBCs membranes. In this article we are not studied this questions in details.

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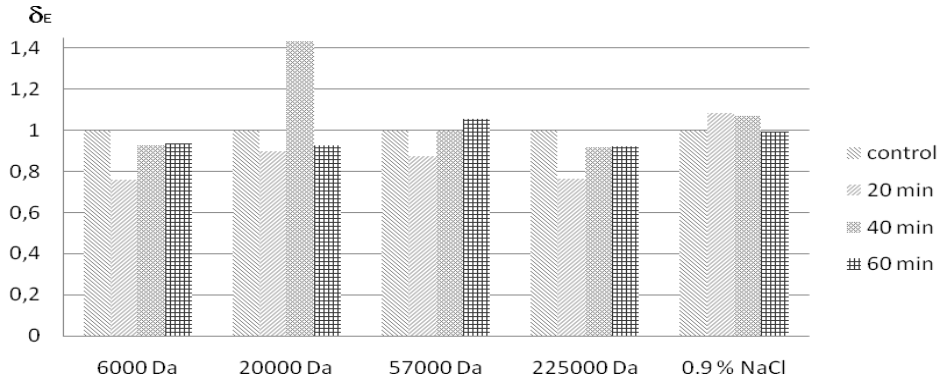


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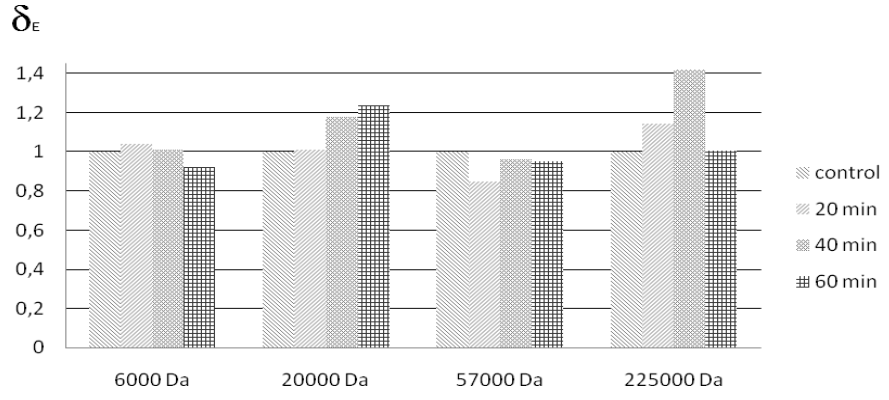


*c*

Fig. 3. Relative elasticity modulus of RBCs membranes after different time incubation with PAA species (linear chains (6000, 20 000 and 225 000 Da) and star-shaped NP (57 000 Da): a – men of the first group, b –men of the second group, c – women of the second group

The values for the relative adhesion force were calculated by formula (2).

(2) $$\delta_F = F/F_{contr}$$

$F$ - adhesion force of RBCs membranes sample after incubation with NPs;
$F_{contr}$ - adhesion force of RBCs membranes control sample

Interesting facts were established by changing local adhesion force values, which are presented on Fig. 4.

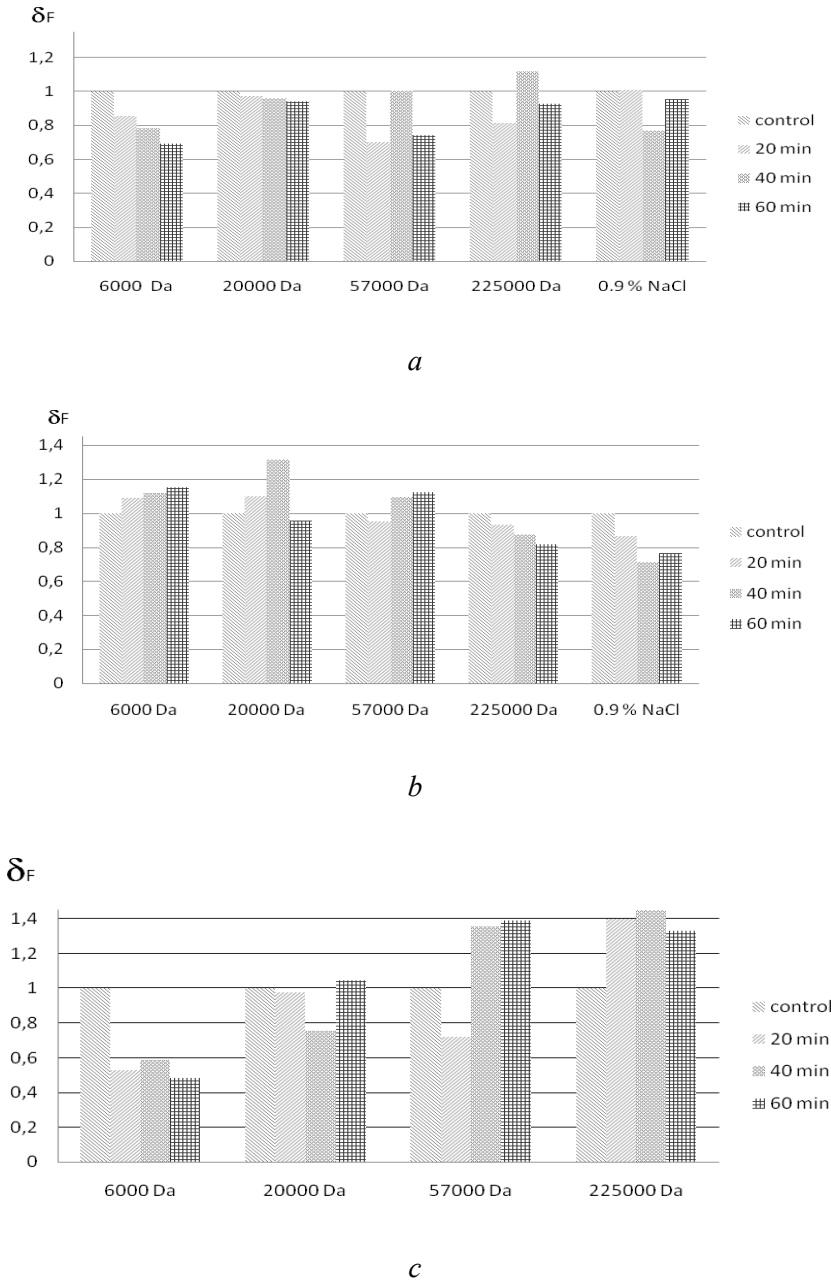


Fig. 4. Relative adhesion force of RBCs membranes after different time incubation with PAA species (linear chains (6000, 20 000 and 225 000 Da) and star-shaped NP (57 000 Da): a – men of the first group, b –men of the second group, c – women of the second group

For the men of the first group adhesion greatly increased after incubation RBCs with PAA NP $M_n$ = 57 000 Da and linear polymer with $M_n$ =225000 Da and decreased after incubation with PAA with $M_n$ = 6 000 Da. For men of the second group such changes were not found. For women's RBCs changes were established after cells incubation with PAA with $M_n$ = 6 000 Da. Local adhesion force decreased monotonically. Unlike elasticity modulus after incubation with physiological solution adhesion force decreased slightly, it is due to change in charge on the surface of the cell membrane.

Additionally each group of patients was divided into two subgroups: patients with chronic kidney disease (CKD) 1 and 2 grade and other group with CKD 3*a* grade.

Table 1

Data for biochemical research

| Patient's # | ESR | HbA1c | TC | TG |
|---|---|---|---|---|
| CKD 3 *a* | | | | |
| 1 | 0,74 | 6,4 | 5,6 | 2,1 |
| 2 | 0,78 | 8,3 | 5,3 | 1,8 |
| 3 | 0,66 | 8,4 | 5,4 | 1,6 |
| 4 | 0,43 | 9,4 | 6,9 | 1,5 |
| 5 | 0,17 | 9,1 | 6,4 | 1,2 |
| CKD 1 and 2 | | | | |
| 6 | 0.43 | 6.3 | 6.3 | 3.4 |
| 7 | 0.43 | 6.3 | 4.5 | 1.1 |

There was a trend that decreasing of ESR was accompanied by decreasing of triglycerides level. Also, in some cases ESR had the same value for each of the subgroups with the same characteristic of HbA1c. The most common examples are listed in Table 1.

## 4. Conclusions

The study of the effect of polymer species with linear and spherical shapes (nanoparticles) on the properties of RBCs membranes in two groups of patients with DM2 has shown that significant changes in the structure of RBCs membranes did not occur and traces of PAA macromolecules or NPs were not found. Certain changes of elasticity modulus were observed after cells incubated with PAA with $M_n$ =20000 Da and 225000 Da, due to their interaction with the components of the cell membrane. After incubation with physiological solution adhesion force decreased slightly, but local elasticity modulus were not changed. Also, it was shown that values of ESR and TG decreased in groups of patients with TC level less than 7.

Thus, these results allow us to give recommendations on the using of these particles in drug delivery. The star shaped NPs with $M_n$ = 57000 Da and bearing arms of PAA lead to the smallest changes in the structure and properties of cells membrane, and it can be expected no conversion of the transport properties of the cells.

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