**Combinatorial screening of polymer nanoparticles for their ability to recognize epitopes of AAV-neutralizing antibodies**

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A library of 17 nanoparticles made of acrylate and methacrylate co-polymers is prepared, characterized and screened against six epitopes of AAV-neutralizing antibodies to assess their affinity and specificity. Peptide epitopes are immobilized onto the surface of glass beads, packed in filtration microplates, and incubated with fluorescein-labelled nanoparticles. Following intense washing, the affinity of nanoparticles to immobilized epitopes is assessed by measuring the fluorescence of captured nanoparticles. The results show that polar monomers, acrylic acid in particular, have a positive impact on polymer affinity towards all peptides used in this study. The presence of hydrophobic monomers, on other hand, has a negative impact on polymer binding. The composition of peptides used in this study has no noticeable impact on the affinity of synthesized nanoparticles. The affinity of nanoparticles with the highest affinity to peptide targets does not exceed millimolar level. Overall, it is found that the synthesized library showed modest affinity but lacked specificity, which should be further ‘tuned’, for example by using molecular imprinting to achieve an acceptable level of affinity and specificity for practical application.

**1. Introduction**

The main objective for the work presented here is the development of a tool for improving the efficiency of gene therapy used in the treatment of Crigler-Najjar (CN) syndrome. CN syndrome is a genetic disorder caused by an inability or diminished ability to convert and clear bilirubin from the body. The inability to convert bilirubin from its unconjugated to its conjugated form is due to a genetic mutation of the enzyme UGT1A1 (Uridine Diphosphate Glucuronosyltransferase 1A1), which is responsible for the conversion. The unconjugated form, which is not readily water-soluble, accumulates in the body and leads to abnormally high levels of the unconjugated form. This toxic substance then builds up in the body, causing unconjugated hyperbilirubinemia and jaundice. This poses threats to the health of the individual and can cause devastating effects as once accumulated, the unconjugated form acts as a neurotoxic on the brain and CNS, causing symptoms such as muscle spasms, fatigue and vomiting, as well other sensory and physical ailments.1 Mutations at one of the genes of the converting enzyme UGT1A1 consequently lead to either a decrease or cessation in the production of the water-soluble form of bilirubin.

Current treatment for Crigler-Najjar syndrome involves phototherapy, liver transplant and genetic therapy. The gene therapy option for treating CN syndrome relies on the use of adeno-associated viruses (AAV) vectors for the delivery of the active UGT1A1 gene into the cells, thereby reversing the effects of CN. The lack of pathogenicity and the perseverance of the virus along with several available serotypes makes AAV the ideal vector for *in vivo* gene therapy. There are, however, limitations to the use of AAV in gene therapy due to vector immunogenicity and pre-existing immunity to AAV.2

These are the major hurdles which require a solution before AAV vectors can become the preeminent form of treatment for inherited conditions. One problem with this route of treatment is that patients have to undergo dialysis/plasmapheresis to remove antibodies from their bloodstream that are produced as a result of an evoked immune response by their body to AAV particles. During this procedure, blood is removed from the patient and their blood cells are separated from their plasma, which contains the antibodies neutralizing AAV-virus vectors. The commercial plasmapheresis columns, which are used in dialysis, remove all antibodies, both neutral and virus-specific. A potential solution would entail developing tools capable of specific removal of AAV-neutralizing antibodies by using immunoadsorbents.3 Unfortunately the costs of treatment using immunosorbents would be too high for this application.

Conversely, synthetic polymeric nanoparticles (NPs) with an intrinsic affinity for specific peptides and proteins represent a new research tool for biological and biomedical research, and may assist with the treatment of many common hereditary diseases by removing antibodies with specificity.4,5 NPs offer a robust matrix, less susceptible to the factors that would render biological matter ineffective, such as high temperature and variations in pH or storage conditions. Due to their relatively low cost and high stability, polymer nanoparticles appear to have a clear advantage over antibodies.

Recently, Shea and colleagues have used combinatorically designed polymers for selective targeting of the toxin peptide melittin and vascular endothelial growth factor VEGF.6,7 Following the same strategy, a small combinatorial library of polymeric nanoparticles was prepared and screened against six peptide epitopes of the variable regions of the AAV-neutralizing antibodies. The monomers used for the synthesis of the NPs are similar to these previously used in Shea’s work (Figure 1).7 Each of the NPs also contained a polymerizable fluorescein (Figure 1) which was incorporated into the backbone of the polymeric matrices. It allowed a characterization of NPs as well as an assessment of binding in the fluorescent microplate assay.

**MATERALS AND METHODS**

**2.1 Materials**

*N*-isopropylacrylamide (NIPAm), *N*-tert-butylacrylamide (TBAm), *N,N′-*methylenebisacrylamide (BIS), acrylic acid (AAc), *N*-phenyl acrylamide (PAm), ammonia persulfate (APS), sodium hydroxide, toluene, *N*-hydroxysuccinimide (NHS), iodoacetic acid *N*-hydroxysuccinimide ester (SIA), ethylenediaminetetraacetic acid (EDTA), 1,2-Bis(triethoxysilyl)ethane, *N*-[3-(trimethoxysilyl)propyl]ethylenediamine, Tween 20, bovine serum albumin (BSA) were purchased from Sigma-Aldrich, UK. *N*-(3-aminopropyl)methacrylamide hydrochloride (APM) was bought from PolySciences, UK. Polymerizable fluorescein *N*-ﬂuoresceinylacrylamide (FlAm) was provided by Leicester Biotechnology Group. Tablets of phosphate buffered saline (PBS) were purchased from Life Technologies, UK. Absolute ethanol and acetonitrile were purchased from Fisher Scientific, UK. Acetone was obtained from VWR International, UK. The glass beads (Spheriglass A glass 2429 CP-00) were bought from Potters, UK. The dialysis was done using Slide-A-Lyzer Dialysis kit, 3-12 mL capacity, membrane cut-off- 10 kDa (Thermo Scientific Ltd, UK). Fluorescence was measured using microplate fluorescence reader Hidex Sense (LabLogic Systems Ltd, UK).

Six peptides (Table 1) representing the surface epitopes of the variable regions of AAV8-netralising antibodies were identified using molecular imprinting by following a protocol developed by Leicester Biotechnology Group and AAV-specific antibodies and non-specific antibodies provided by the AFM-Telethon laboratory Genethon (France).8 The peptides were custom-made by Zhejiang Ontores Biotechnologies Co., Ltd, China. All peptides were of >95% purity and contained one cysteine amino acid on the amino end of the peptide, which was used for immobilization on the solid phase via thiol coupling. The selected peptides differed by their size, polarity and charge in order to maximise the chances of identifying NPs with enhanced affinity to particular peptide sequences (Table 1).9

**2.2 Synthesis of NPs**

The typical protocol for synthesis of nanoparticles is as follows. NIPAm (143 mg, 1.26 mmol), BIS (5 mg, 0.032 mmol), AAc (10 µL, 0.16 mmol) and APM (27 mg, 0.15 mmol), were weighed out and dissolved in high purity distilled water (25 mL). The compositions of all nanoparticles are shown in Table 2. Fluorescent monomer fluorescein acrylate (0.8 mg) was dissolved in 200 μL of neat ethanol and added to the monomeric mixture. The solution was then transferred to a single-neck round-bottomed flask (50 mL) with a magnetic stirring bead and purged with nitrogen for 30 min. APS initiator (15 mg) was dissolved in 1 mL of distilled water and added to the monomer mixture using a syringe. The reaction flask was placed onto a hotplate stirrer pre-heated to 60 °C. The reaction mixture was left to polymerize at 60 °C with gentle stirring for 2.5 h. All NPs were synthesized using the same protocol. 12 mL of each synthesized nanoparticles were placed into the dialysis cartridges with a molecular weight cut-off of 10 kDa and were purified from non-reacted monomers against 1.5 L of water for one week with regular (once a day) changes of water.

**2.3 SEM analysis of nanoparticles**

SEM analysis was performed using a Zeiss Leo Gemini 1525 (Zeiss, Germany). Nanoparticles were diluted to 1 µg mL-1, sonicated for 1 min, 20 µL were added to a sample plate, and the plates coated with chromium for 3 minutes under vacuum prior to imaging. Statistical analysis was conducted by measuring the randomly selected particles using open source ImageJ program.

**2.4 Preparation of glass beads with immobilized peptides**

The 120 g of solid phase glass beads (7 - 25 μm diameter) were placed into a flask with 4 M NaOH, boiled for 15 minutes, rinsed with deionized water, 500 mL of PBS and thoroughly washed again with deionized water. Beads were washed with acetone and left to dry under vacuum. Finally, beads were placed into a pre-heated oven at 150 °C for half an hour. Dried glass beads were placed into 50 mL of hot toluene and 5 mL of *N*-[2-(aminoethyl)-3-aminopropyltrimethoxysilane was added. The mixture was left to react for 24 hours at 70 °C. The silanized beads were then filtered and washed with eight volumes of acetone (8 x 100 mL). The beads were then dried under vacuum and placed in the oven at 150 °C for two hours. 10 mg of iodoacetic acid *N*-hydroxysuccinimide ester (SIA) was added to 120 g of the silanized glass beads in acetonitrile (50 mL) and incubated in the dark for 2 hours. After incubation, the beads were washed with acetonitrile (5 x 100 mL). 10 mL of thiol buffer containing 5 mM of EDTA in PBS, pH 8.2, was added to the 25 g of solid phase and purged with nitrogen for 20 min. The solution of peptide (5 mg) in 200 μL of DMSO was adjusted with water to 1 mL and added to the glass beads in PBS. The mixture was left for incubation overnight (≈20 h) in the dark. The solid phase with immobilized peptide was rinsed with high purity water (1.5 L) to remove any by-products and dried in vacuum.

**2.5 Analysis of NPs binding**

The assay was carried out in a 96-well filter microplates (Agilent Technologies, USA). Microplate wells were packed with 50 mg (± 5 %) of the solid phase with immobilized peptide. 100 μL aliquots of the blocking solution containing 0.1% of BSA (1 mg mL-1) and 1% Tween 20 in PBS was pipetted into wells and left to incubate for 10 minutes. After the removal of the blocking solution the microtitre plate was loaded with fluorescent NPs (100 μL, 10x dilution in PBS) and incubated for a further 30 minutes. The concentration of the nanoparticles was normalized accordingly to the fluorescence intensity and was on average 40 μg mL-1. The sample was then filtered and the fluorescence measured using a Hidex microtitre plate reader. The fluorescent parameters were set as λex=485 nm and λem=575 nm. The microtitre plate was then washed using PBS (100 μL per well) using multichannel pipette (Socorex Isba S.A., Switzerland) and filtered. This step was repeated 3 times. The remaining fluorescence was then analyzed using a multimodal microplate reader. All measurements were made in triplicate.

**2.6 Measurement of NPs affinity by Biacore**

The interaction analysis was performed using the Biacore 3000 instrument (GE Healthcare Life Sciences, UK) at 25 °C using PBS (0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4) as the running buffer at flow 35 µL min-1. The self-assembled gold sensor chip (SA) has been cleaned using plasma and placed in the ethanol solution of mercaptododecanoic acid (2.2 mg mL-1) where they were stored until use. Before assembly, the sensor chip was rinsed with ethanol and water, and dried in a stream of air. The selection of nanoparticles for the Biacore experiment was based on the availability of amino groups in their composition (10 mol% of APM) and results of the microplate screening assay suggesting specificity of NP10 for peptide 4 in the screening experiments. The nanoparticles NP10 were x100 diluted in PBS (4 μg mL-1), sonicated in the ultrasonic bath for 10 min and then injected and immobilized in situ on the chip surface containing carboxyl groups using the EDC/NHS coupling (0.4 mg and 0.6 mg per milliliter, correspondingly). The solution of peptide 4 was diluted in PBS in the concentration range between 46 nM and 0.46 µM. Sensorgrams were collected sequentially for all nanoparticles concentrations running in KINJECT mode (injection volume- 100 μL and dissociation time- 120 sec). The dissociation constant (KD) was calculated from plots of the equilibrium biosensor response using the BiaEvaluation v.4.1 software in the 1:1 binding Langmuir Blodgett (LB) model using the AB (absorption) component of the SPR obtained after the subtraction of the drift and bulk effects.

**2.7 Statistical analysis of data**

To evaluate the stability or precision of measurements, we calculated the coefficient of variation (CV).10 CV calculated by the formula

where is sample estimation of standard deviation and is mean value of binding. CV characterises relative variations and can be applied for ratio scale data.11 To check the significance of differences we have used Student’s t-test. As a result of the small sample size we used s significance level of 90%. In this study we used two sample t-test with unequal variances, using the formula

where is mean value of sample, is variance of sample, is size of sample. The number of degrees of freedom for t-distribution is

P-value of test is calculated as

where is the cumulative distribution function of t-distribution with degrees of freedom for statistics .

RESULTS AND DISCUSSION

There are several ways of designing polymeric binders for small molecules, peptides and proteins. These include combinatorial and computational designs of polymeric adsorbent from a library of functional monomers.12,13 So far, computational design of polymeric adsorbents has proven to be more successful for small molecules, but not peptides or proteins. The reason for this lies in small variations in their structure and the large number of polar domains in protein structure that cannot be discriminated by virtually- designed polymers in molecular modelling experiments.13

The combinatorial design used in Shea’s group was more successful. The study entailed the screening of a polymer library in the development of polymer nanoparticles capable of recognizing targets such as melittin and VEGF.6,7 The authors have used systematic variations in the ratio of functional monomers for the synthesis of polymer nanoparticles, which were then screened for their affinity to the target molecules. These monomers have functional groups that are able to effectively capture and neutralize the toxicity of the peptide through a combination of electrostatic, hydrophobic, hydrophilic and hydrogen bonding interactions. The study culminated in an identification of nanoparticles with high affinity (0.6 μM to 2.1 μM) for selected peptides and proteins.14 The results from the study showed that these synthetic receptors did show significant binding to the melittin and thus could prove an effective alternative to the more complex forms of treatment that involve antibodies. Nanoparticles in these experiments were synthesized by precipitation polymerization from a diluted solution. The monomers used for the synthesis of the NPs are similar to those used previously7 and include NIPAm, positively charged APM, negatively charged AAc, hydrophobic TBAm and aromatic monomer PAm. BIS acted as the cross-linker (see Table 2). Each of the NPs also contains polymerizable fluorescein used as a fluorescent reporter for the characterization of the NPs as well as the assessment of binding in the fluorescent microplate assay.

Following this protocol, we have synthesized 17 nanoparticles and assessed them on their ability to bind to six peptide epitopes of AAV-neutralizing antibodies. The synthesis of NPs was performed in a dilution solution of monomers as described previously,6 except that the use of surfactant in polymer preparation was avoided. In our experience, the presence of surfactant is not required and, in addition, it is practically impossible to remove it quantitatively from synthesized polymers. The presence of surfactant can also affect nanoparticles’ biocompatibility of blood cells.

The yield of synthesized nanoparticles was consistent, varying from 55 to 140 mg, depending on the nanoparticles composition. It was found that the monomeric mixture containing polar monomers produced a lower yield of nanoparticles than the monomeric mixture containing hydrophobic monomers. From the fluorescence analysis it was clear that fluorescein monomer was incorporated into all NPs (see Fig 1S in Supplementary Information section). Fluorescein has an emission peak at 515-520 nm, and all samples of various compositions had an emission within the range of 510-518 nm.

The difference in fluorescence from sample-to-sample was quite substantial, varying from 8 Million RFU to 18 Million RFU, depending on the composition of polymers. According to SEM measurements, the size of nanoparticles varied from 23.0 to 43.5 nm (Figure 2). The statistical information on analysis of the nanoparticles diameter is shown in the Fig. 2S-7S, Supplementary Information). All nanoparticles were aggregating in the solution and for this reason we had to sonicate all samples before each binding experiment. It is important to note that the same aggregation is also observed for nanoparticles prepared in the presence of surfactant, which had little impact on their stability in the solution. For future application, aggregation can be reduced by the surface functionalization of synthesized nanoparticles using PEGylation.15

The binding of nanoparticles to immobilized peptides was assessed by measuring the difference in fluorescence of NPs adsorbed onto the solid phase before and after the washing. All experiments were performed in a buffered solution at physiological pH to avoid the impact of peptide charge on the fluorescence of NPs. Conveniently, adsorption, washing of NPs and measuring the level of the remaining fluorescence were all performed directly using filtration microplates in the reflectance measurement mode. The filtration microtiter plate format allowed to incubate the packed solid phase with immobilized peptides in the wells, incubate them with corresponding solution (either blocking solution, containing nanoparticles or washing solution depending on the experiment step), remove it by filtering using a vacuum manifold and measure the remained fluorescence of the dry solid phase with bound nanoparticles. Figure 3 shows changes in the fluorescence of bound nanoparticles during washing. Three washing cycles were considered sufficient for a qualitative comparison of NPs binding to glass beads with immobilized peptides.

The results of the NPs binding to glass beads with the immobilized peptides are presented in Figure 4. Overall, the binding, which was calculated as a percentage of fluorescence left in a microplate after the washing, varied from 10% to 70%.

We can see that the composition of nanoparticles has indeed had an impact on the ability of nanoparticles to bind to peptides. It is clear that the presence of polar monomers such as AAc and APM had a positive impact on binding, as compared with N17 that does not contain functional monomers, while hydrophobic monomers such as TBAm and PAm had negative effect on binding. Interestingly, the addition of a positively charged monomer (APM) to the composition containing AAc had an adverse effect on the binding of most NPs to peptides.

We used statistical analysis, in particular, the coefficient of variation (CV), to further analyze the binding data produced. The degree of binding is a clearly ratio scale. CV characterizes variability, which is a combination of two factors: experiment reproducibility and precision of measurements. Under the assumption of good reproducibility of experiments and uniform error distribution, a CV of 10% corresponds to an error of measurement of 17%. Under the assumption of symmetric triangular distribution, a CV of 10% corresponds to an error of measurement of 24%. Results presented in Table 3 show that, on average, the coefficient of variation was 6.1% in our experiments. Furthermore, there are 16 pairs with a CV greater than 10%, and 3 pairs with a CV greater than 20% (Table 3).

As a result of the low CV observed in most cases, we can conclude that experimental reproducibility is only questionable in limited specified pairs. Table 4 shows the significance of difference in binding between N17, which does not contain functional monomers, and the remaining nanoparticles. We can see that for most particles, the influence of monomer composition on binding to all six peptides is very similar: an increase in binding for all peptides with respect to N1–N8 and a decrease in binding for all peptides with respect to N11 – N16. N10 demonstrates specificity for peptides P4, P5 and P6 together and only N9 demonstrates specificity to one peptide, P6. The data in Table 4 shows that 56 out of 96 pairs of peptide/NP combinations have a negative or neutral effect, as compared to N17. For 40 peptide/NP combinations, the monomer composition of the NPs has a statistically significant positive effect. Only N9 out of all NPs have enhanced binding to one peptide only (P6). Even in this case, the difference in binding is quite small. It is possible to confirm that there were no nanoparticles specific only for P1, P2, P3, P4 or P5. The best binding nanoparticles (N7) have a similar composition to the one found in Shea et al.’s experiments.6  Since there was no real discrimination of the binding of N7 to six peptides, we can assume, however, that this effect of composition is not specific.

To evaluate the affinity between the NPs and peptides, we have tested binding between NP10 and peptide 4 using Biacore 3000 (GE Healthcare, UK). NP10 were immobilized onto the gold surface of Biacore chips using EDC/NHS coupling, and several different concentrations of the peptide 4 (0.46 nM- 46 µM) in PBS were injected. The results of these experiments (see Figure 5) show that the average NPs/peptides pair does not have an affinity exceeding 100 μM (Chi2= 9.12E-06).

The binding of the nanoparticles was most likely compromised by their fast aggregation leading to a reduction in the affinity due to steric limitations of large agglomerates.16 However, even if binding can be further improved by 1-2 orders of magnitude by reducing the aggregation using PEGylation, the binding would most likely still be too small for a practical application in the specific removal of AAV-neutralizing antibodies by plasmapheresis. We believe that a way to obtain nanoparticles with a high affinity for application in separation would be to produce nanoparticles using molecular imprinting.17 Recently, very successful MIP nanoparticles with a sub-nanomolar affinity were prepared for a range of peptides and proteins using precipitation polymerization, grafting and solid-phase imprinting.18-20 We intend to test a combination of combinatorial selection of monomers and molecular imprinting in the future for the extension of the present work.

To summarize, we can conclude that acrylic acid positively influenced NP’s binding to peptides. 5% of AAc was sufficient, and no further improvement in binding was observed upon further increase in AAc concentration. The addition of the hydrophobic monomer (TBAm) did not affect specific binding of AAc-containing nanoparticles to peptides. The presence of the hydrophobic monomer (TBAm) has reduced binding between the peptides and nanoparticles containing 5% AAc, and slightly increased it when nanoparticles contained 10% and 20% of AAC interacted with peptides P4, P5 and P6. It was observed that the addition of a large quantity of positively charged monomer (APM) decreased the binding of AAc-containing nanoparticles to peptides. The optimum ratio was identified as 5% AAc and 10% APM. Replacing AAc with the hydrophobic monomer PAm significantly decreased binding of NPs to the peptides. No obvious effect of composition, length or charge of the peptides was observed on the binding to NPs. Therefore, it is not possible to conclude that any NP in the combinatorial library was sufficiently specific to each particular peptide.

**CONCLUSIONS**

A combinatorial library containing 17 batches of nanoparticles with different compositions was produced and characterized. In order to select the fluorescent nanoparticles which demonstrate affinity interactions with six peptides of interest, a novel high throughput screening method was developed. The peptides were immobilized on the glass beads, packed in the filtration plates and their binding to the library of fluorescent nanoparticles was tested using a standard microtiter plate. The precision of the measurements was evaluated using a coefficient of variation algorithm. Overall, it was found that the synthesized library of nanoparticles showed a modest affinity but lacked specificity, which should be ‘tuned’ by using molecular imprinting to achieve acceptable levels of affinity and obtain specificity required for practical applications.18

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

**AUTHOR CONTRIBUTIONS**

EVP- training and supervision of the project, corresponding author; EM provided mathematical calculations; SSP- SEM imaging and characterisation of the nanoparticles; SAP- project idea, supervision and preparation of the manuscript; FG- training and supervision of students; FM- peptides acquisition and discussions. The manuscript was prepared in the frame of the BSc research project in the School of Chemistry, University of Leicester. Combinatorial library of seventeen nanoparticles was made by a group of ten students with equal contributions in relation to preparation of the corresponding nanoparticles and their characterization- HA, AC, EC, SD, SJE, HF, AH, MO, NP, AS.

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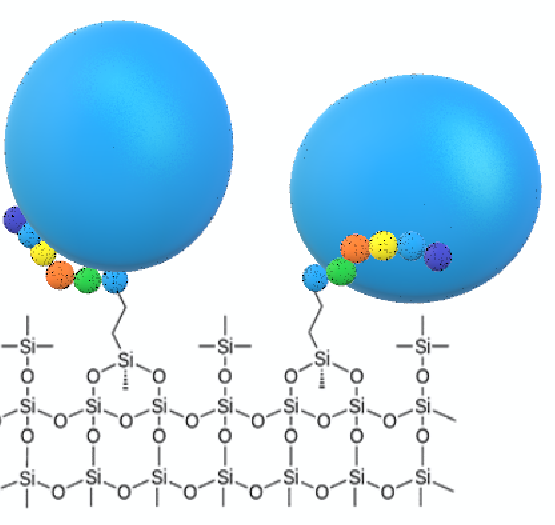
A small combinatorial library of polymeric nanoparticles was prepared and screened against six peptide epitopes of the variable regions of the AAV-neutralizing antibodies following a protocol developed by the leader in this field, Prof Ken J. Shea. It addresses previously unanswered questions, particularly in relation to whether synthesized nanoparticles have not only affinity, but also specificity to different peptide/protein modalities.

Keywords: nanoparticles, affinity, antibodies, epitopes, molecular imprinting

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ToC graphics



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Supporting Information

Combinatorial screening of polymer nanoparticles for their ability to recognize epitopes of AAV-neutralizing antibodies

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Supplementary Information section contains the typical fluorescent spectrum of the nanoparticles (NP1) which demonstrates the inclusion of FlAm in the polymer matrix (Fig. 1S) and SEM images of all five groups of combinatorial library of nanoparticles (Fig. 2S-6S) and SEM image of the control sample of nanoparticles (NP17) which was made without functional monomers (Fig. 7S).

Figure 1S. Fluorescence emission of N10 measure at λex= 485 nm and λem range between 500 and 600 nm.



Figure 2S. SEM image of the nanoparticles 4; inset picture -statistical analysis of the nanoparticles 4 size. The size of nanoparticles 4 calculated using ImageJ software is 23.0 ± 3.6 nm.



Figure 3S. SEM image of the nanoparticles 7; inset picture -statistical analysis of the nanoparticles size. The size of nanoparticles 7 calculated using ImageJ software is 43.5 ± 10.7 nm.



Figure 4S. SEM image of the nanoparticles 10; inset picture - statistical analysis of the nanoparticles size. The size of nanoparticles 10 calculated using ImageJ software is 27.0 ± 4.4 nm.



Figure 5S. SEM image of the nanoparticles 13; inset picture -statistical analysis of the nanoparticles size. The size of nanoparticles 13 calculated using ImageJ software is 23.6 ± 3.9 nm.



Figure 6S. SEM image of the nanoparticles 16; inset picture - statistical analysis of the nanoparticles size. The size of nanoparticles 16 calculated using ImageJ software is 23.8 ± 4.7 nm.



Figure 7S. SEM image of the nanoparticles 17; inset picture -statistical analysis of the nanoparticles size. The size of nanoparticles 17 calculated using ImageJ software is 36.4 ± 5.9 nm.