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# Multi-objective optimization and design of experiments as tools to tailor molecularly imprinted polymers specific for glucuronic acid

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ABSTRACT

We present a multi-objective optimization of the binding properties of a molecularly imprinted polymer (MIP) which specifically binds glucuronic acid (GA). A design of experiments approach is used to improve four different parameters that describe the binding properties of the polymer. Eleven different methacrylamide-*co*-ethyleneglycol dimethacrylate polymers imprinted with GA were synthesized according to a full factorial experimental design plan with 3 influencing factors (degree of cross-linking, molar equivalent of monomer to template and initiator concentration). These polymers were characterized by adsorption of the radiolabeled target analyte in methanol:water 9:1. The binding parameters were computed to optimize the polymer composition, taking into account four objective variables: the maximum binding capacity at high ($B_{max}$) and low ($B_2$) analyte concentrations, the equilibrium constant $K_{50}$, and the imprinting factor (IF, binding to MIP/binding to NIP). With the multi-objective optimization method based on a desirability approach the composition of a twelfth "ideal" polymer could be predicted. This predicted polymer with highest "desirability" was synthesized with a composition of 0.65 mol% of initiator and a 1:4:20 ratio of template:functional monomers:cross-linker (T:M:X) (80% of cross-linking), and found to be the overall best MIP. Improvements over the original starting polymer were a 6 times lower $K_{50}$, which corresponds to higher affinity, 20% higher capacity at low analyte concentration ($B_2$), 40% higher capacity ($B_{max}$) and 1.3 times increased imprinting factor (IF). Binding assays were also performed in aqueous solvents. Good binding properties were obtained in pure water with an imprinting factor of 3.2. Thus, this polymer is potentially applicable to biological samples like urine where glucuronides occur.



## 1. Introduction

In the field of bio- and chemosensing for food, environmental and biomedical analysis there is a continuous need for appropriate recognition elements that specifically detect a target of interest. The requirements for these sensors are dependent on the application, e.g. thermal and chemical stability, reusability, rapidness, high sensitivity, selectivity and specificity [1,2]. In medical diagnostics important targets are sugar derivatives, such as glycosylation sites on cell surfaces like cancer cells [3] or on infectious bacteria [4], as well as glycoconjugate drug metabolites [5].

There is still a lack of suitable biological recognition elements for the sensitive and selective detection of these molecules in technological applications. However, molecularly imprinted polymers (MIPs) are promising synthetic receptor materials in this context. Molecular imprinting of polymers relies on the presence of a molecular template during polymerization that directs the self-assembly of monomers carrying suitable functional groups. In an excess of cross-linking monomers, the functional monomer copolymerize forming a cast-like shell around the template, and subsequent removal of the latter liberates three-dimensional binding sites in the material that are complementary to the template in size, shape and position of the functional groups. Molecularly imprinted polymers are suitable as sensor materials since they are robust, reusable, and often exhibit excellent binding properties, with affinities and selectivities in some cases comparable to antibodies [6]. Therefore, they are sometimes called 'antibody mimics' [7].

The molecular imprinting of glycoconjugates of biomacromolecules, in particular of proteins, is a challenge since their conformation is very sensitive to solvent, temperature, pH and

*Abbreviations:* AAB, N-acrylamido-benzamidine; AIBN, azobisisobutyronitrile; EDMA, ethylenedimethacrylate; GA, Glucuronic acid; MAM, methacrylamide

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ionic strength [8]. Researchers have therefore suggested to use the so-called 'epitope approach', that is, to imprint a structural epitope of the target molecule of interest, rather than the whole molecule, in order to obtain a synthetic receptor able to recognize both the low molecular weight template and larger molecules that possess the template as terminal part [9]. In the present work, we have chosen to create molecular imprints of a small monosaccharide unit, glucuronic acid, as an epitope of a number of glycoconjugates. Glucuronic acid is part of the glycocalix or intercellular matrix where it mainly can be found as a component of hyaluronan. Recently the group of Sellergren has reported molecularly imprinted polymers tailored with urea functionality against lipophilic derivatives (1,2,3,4-tetra-O-acetyl and 1-O-dodecyl) of glucuronic acid. Glucuronated metabolites could be recognized by the resulting material in a solid-phase extraction (SPE) process in a water containing solvent [10,11].

In our study the template glucuronic acid was used without any derivatization for molecular imprinting. Imprinting underivatized glucuronic acid is a challenge, since both the self-assembly of the template–monomer complex and the recognition of the target by the MIP will have to take place in a polar environment, which is not favorable for the establishment of non-covalent interactions. In addition, we wanted to perform the binding experiments at equilibrium, where the affinity of the sites is even more important compared to chromatographic analysis where multiple association–dissociation phenomena can take place [12].

The development and optimization of a MIP for a particular target can be rather complex as it depends on a large number of variable factors (compositional and operational influencing factors) which are to some extent even dependent on each other. Especially sugar imprinting is not straightforward because of the strong polarity of these molecules and the tendency of their hydroxyl groups to interact with water, if present in the medium, which can interfere with the imprinting process and during recognition of the target by the MIP. Therefore, it is an advantage to perform this optimization with a chemometrics approach like design of experiments (DoE) [13,14], computational methods that model chemical interactions between the target molecule and functional monomers by computer simulations [15] or combinatorial methods for high-throughput synthesis of chemical libraries which can also be coupled with DoE [16,17]. Overviews about these polymer optimization methods are given in [18–20]. DoE is a systematic optimization method for the identification of significant factors that have influence on the process in question, and the modeling of the response with a minimal number of experiments for a determined statistical certainty.

The optimization of molecularly imprinted polymers with experimental design, the approach that we have chosen for the present work, was done for the first time in 2003 by Davies et al. and Navarro-Villoslada et al. [21,22]. To date, only about one dozen publications illustrate the power of DoE for MIP optimization. Different factors and their combinations influencing the analytical properties of the polymers were considered like: the amount of template/monomer(s) [16,22–24], the type of cross-linking monomer [22,25], the influence of the initiator [16,23] and different porogenic solvents [22] as well as the polymerization initiation method (thermal/UV) [16,22]. The types of experimental designs used vary depending on the application: full factorial design [21], central composite design [24,26], fractional factorial design [16] or Doehlert's second order uniform shell design [25]. Since usually many influencing factors are under investigation, a multivariate data analysis is often performed to find cause-effect correlations that are then used to find the optimum polymer composition. To our knowledge, more than one objective parameter was never considered in order to find that composition. This is surprising because to describe the binding properties of a MIP, multiple parameters like affinity, capacity, specificity and selectivity are important.

Here, a multi-objective optimization of the binding properties of a molecularly imprinted polymer on the example of a MIP specific for glucuronic acid is presented for the first time. DoE is used to create the experimental table that corresponds to different MIP compositions representing the design space and to optimize the design variables. The different polymers were characterized by their binding of the radiolabeled target analyte in a methanol:water 9:1 solution in order to compute the objective as well as the response surface functions. The aim of this study, apart from obtaining a MIP for glucuronic acid, is to show that the multi-objective analysis coupled with DoE is a highly promising tool for the optimization of imprinted polymers. The proposed strategy relies on the improvement of 4 dependant variables describing the polymer binding properties: the maximum binding capacity at high ($B_{max}$) and low ($B_2$) analyte concentrations, the equilibrium constant $K_{50}$, and the imprinting factor (IF, binding to MIP/binding to NIP) by varying 3 influencing factors (cross-linking degree, molar equivalents of monomer to template and initiator concentrations). Based on the results obtained for target binding in a methanol:water 9:1 environment, the specific binding of glucuronic acid by the MIP in a purely aqueous environment, which is more relevant with respect to biological samples, was also evaluated.

## 2. Materials and methods

### 2.1. Chemicals

All chemicals were purchased either from Sigma-Aldrich (St-Quentin Fallavier, France) or VWR International (Fontenay-sous-Bois, France), unless otherwise stated. Deionised water was obtained from Milli-Q Gradient (Millipore, France). Methanol (AnalaRNormapur) and acetic acid (GPR Rectapur) were obtained from VWR, hydrochloric acid (Analytical grade, Fisher Scientific, Illkirch, France). Ethylenedimethacrylate (EDMA), methacrylamide (MAM) were obtained from Aldrich, D-glucuronic acid (GA) was from Sigma. Azobisisobutyronitrile (AIBN, or VAZO 64) was from DuPont Chemicals (Wilmington, USA). D-[6-$^{14}$C]glucuronic acid (specific activity: 50–60 mCi/mmol, activity: 0.1 mCi/mL) was from Biotrend Chemikalien GmbH (Germany).

The synthesis of (N-acrylamido)-benzamidine (AAB) was adapted from [27]. AAB was prepared from acryloyl chloride and p-aminobenzamidine. The reaction was conducted in an ice bath by adding 4 ml acryloyl chloride dropwise to 200 mL aqueous solution of sodium acetate (34 g) containing 2 g of p-aminobenzamidine. The addition of acryloyl chloride was completed within 10 min. Afterwards, the reaction was allowed to continue for 1 h. The final product was then precipitated by adjusting the pH to 4 with concentrated nitric acid. After dissolution in hot water, the product was recrystallized by adding dilute nitric acid and then washed with cold water. The compound was dried at 50 °C and the yield was 88.4%.

### 2.2. Polymerization procedure

MAM-*co*-EDMA-polymers imprinted with GA in the presence of AAB were prepared by polymerization in anhydrous DMSO under dilute conditions. The amounts of precursors were determined by the DoE (Tables 1 and 2). Initially, AAB was dissolved in DMSO in a 4 mL glass vial with an airtight septum under sonication (Elmasonic, Germany) for 30 min, followed by the addition of GA. The solution was left to equilibrate on a rotator (Labinco BV, the Netherlands) for 30 min. Then, the other components (MAM, EDMA and AIBN) were added, the final volume adjusted with anhydrous DMSO, and the mixture was purged

**Table 1**
Influencing factors (dependent variables) and their levels.

| Influencing factors | | Factor levels | |
|---|---|---|---|
| Functional monomer | MAM, eq to template | 3 | 7 |
| Degree of cross-linking | CL, % | 60 | 83 |
| Initiator | AIBN, mol% of double bonds | 0.56 | 1 |

**Table 2**
Experimental plan as full factorial design with 3 influencing factors (functional monomer MAM ratio to template, cross-linking degree (CL) and initiator AIBN) with 2 factor levels each. The stoichometric ratio of template (GA): AAB and the total concentration of monomers ($c_m$) were kept constant.

| Run no. | GA, eq | AAB, eq | $c_m$, (%) | EDMA, eq | MAM, eq | CL (%) | AIBN (mol%) |
|---|---|---|---|---|---|---|---|
| 1 | 1 | 1 | 5 | 6 | 3 | 60 | 0.56 |
| 2 | 1 | 1 | 5 | 6 | 3 | 60 | 1 |
| 3 | 1 | 1 | 5 | 20 | 3 | 83 | 0.56 |
| 4 | 1 | 1 | 5 | 20 | 3 | 83 | 1 |
| 5 | 1 | 1 | 5 | 12 | 7 | 60 | 0.56 |
| 6 | 1 | 1 | 5 | 12 | 7 | 60 | 1 |
| 7 | 1 | 1 | 5 | 40 | 7 | 83 | 0.56 |
| 8 | 1 | 1 | 5 | 40 | 7 | 83 | 1 |
| 9–1 | 1 | 1 | 5 | 15 | 5 | 71 | 0.78 |
| 9–2 | 1 | 1 | 5 | 15 | 5 | 71 | 0.78 |
| 9–3 | 1 | 1 | 5 | 15 | 5 | 71 | 0.78 |

with nitrogen for 2 min. Polymerization was performed for 18 h at 50 °C in an oil bath. Sonication with a probe sonicator (Branson Sonifier 250, Branson Ultrasonic Corp., USA) for 1 min at 30% power resulted in a suspension of spherical particles. The polymer suspensions were then transferred to 50 mL centrifuge tubes and washed under agitation with 3 runs of HCl (1 M), 3 runs of methanol:acetic acid (9:1 v/v) and 4 runs of methanol, incubating in each solution for $2 \times 2$ h and $1 \times 18$ h. After each washing step the polymers were separated by centrifugation at 20,000 rpm for 20 min at 15 °C. At the end they were dried under vacuum at room temperature. Non-imprinted polymers (reference polymers, NIP) were synthesized in the same way but without the addition of the glucuronic acid template.

### 2.3. Characterization of the polymer library

For radioligand binding assays, a stock solution of radiolabeled GA was prepared from the commercial solution by dilution in water to a concentration of 16.6 pmol (0.83 nCi) of D-[6-$^{14}$C]glucuronic acid in 0.1 mL. A series of polymer concentrations (0.1, 0.3, 0.5, 0.8, 2.0 mg/mL polymer) was prepared from a stock suspension in methanol in 2-mL polypropylene microcentrifuge tubes. 16.6 pmol of radiolabeled glucuronic acid was added and the samples adjusted to 1 mL with the suitable solvent. The tubes were left at ambient temperature overnight on a tube rotator (Labinco). They were then centrifuged at 17,500 rpm for 30 min at 15 °C, and 0.3 mL of the supernatants were pipetted into scintillation vials that contained 2 mL of scintillation liquid (Ultra Gold, PerkinElmer). The amount of free radioligand was measured with a liquid scintillation counter (Beckman LS-6000 IC, Beckman Instruments, CA). The amount of glucuronic acid bound to the polymers was calculated by subtracting the amount of unbound GA from the initial amount of GA that was added to the mixture. For the determination of $B_{max}$ at high analyte concentration, 3 mg of polymer was incubated with 0.1 mM of GA additioned with 83 pmol of D-[6-$^{14}$C] glucuronic acid in a total volume of 1 mL.

The solvodynamic diameter (Z-average) of the particles was determined by dynamic light scattering (DLS) measurements on a Malvern Instruments ZetaSizer Nano ZS, in the solvent of synthesis. The polydispertsity index (PDI) was calculated from the DLS data based on the cumulants analysis as outlined by the manufacturer (Malvern Instruments). The cumulants analysis is the fit of a polynomial to the log of the G1 correlation function $\ln[G1]=a+bt+ct^2+dt^3+et^4+\ldots$. The coefficient of the squared term, $c$, when scaled as $2c/b^2$ is known as the polydispersity, or polydispersity index. The calculations for these parameters are defined in the ISO standard document 13321:1996 E.

## 3. Results and discussion

### 3.1. Properties of the polymer library

In order to demonstrate the power of a multi-objective optimization by DoE in the context of MIPs, we chose a MIP specific for glucuronic acid as a model. A functional monomer (AAB) capable of strongly interacting with carboxyl groups was used in a stoichiometric ratio with the template in all polymers. The other monomers are MAM as a hydrogen-bonding functional co-monomer and EDMA as cross-linker (Fig. 1).

The polymers obtained by the experimental design showed similar physical characteristics concerning their physical softness and opalescence. The polymer yield was for all syntheses between 64 and 98%. Sonication with a probe sonicator resulted in a suspension of particles in each case. Therefore it can be expected that a single polymer "system" behavior is underlying. The particle size as determined by DLS varies from around 400 nm–820 nm for MIP and from 420 nm–810 nm for the reference polymer (NIP). The polydispersity index of the polymer particles was $<0.23$ when determined by DLS in methanol. Solvent exchange from DMSO to methanol results in a decrease in the particle size by a factor of 1.5–2.2 depending on the polymer, although no clear correlation between the swelling and the polymer composition was found.

Before analyzing the polymer library of the DoE, the reproducibility of the radioligand binding experiments with liquid scintillation counting was verified. The relative standard deviation of the method with 0.5 mg polymer and 16.6 pmol glucuronic acid (GA) is $+/-4.6\%$ (5 times replicate measurements of the

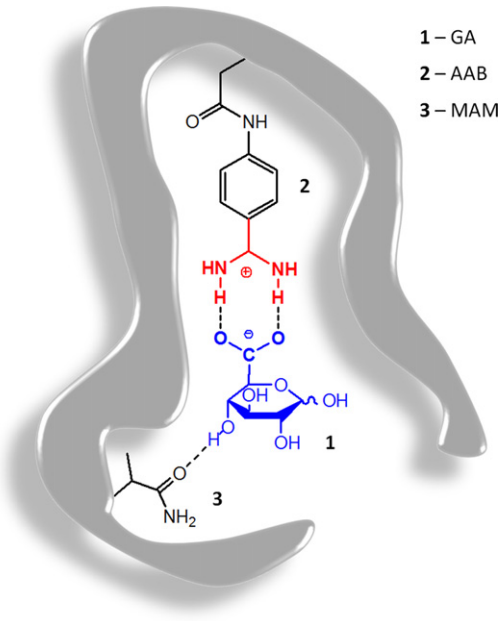


**Fig. 1.** Schematic representation of a molecularly imprinted polymer binding pocket with the template glucuronic acid (1) and the monomers (N-acrylamido)-benzamidine (AAB) (2) and methacrylamide (MAM) (3).

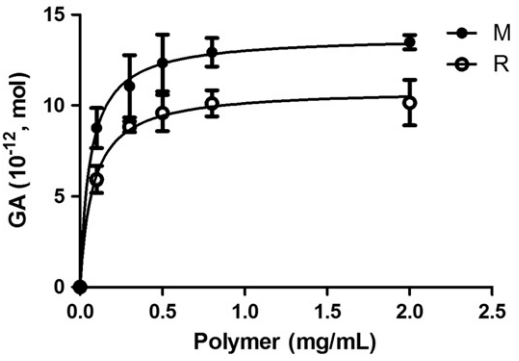


**Fig. 2.** Equilibrium binding isotherms of polymer no 9 in methanol:water 9:1, recorded with 3 independent polymerization reactions and 3 independent binding experiments each; M — imprinted polymer, R — reference (non-imprinted) polymer.

supernatant of the same sample). Furthermore the reproducibility of the polymerization procedure was determined with the center point polymer (no 9) which was synthesized three times independently. Evaluation by radioligand binding assays (Fig. 2) yields an absolute standard deviation of 9.6%.

### 3.2. Experimental design plan and regression analysis

The experimental design plan was created out of 3 *influencing factors* ($x_1$, $x_2$, $x_3$) (Fig. 3):

- MAM–the molar ratio between functional co-monomer MAM and template (which thus corresponds to different relative amount of template present in the system)
- CL–the degree of cross-linking
- AIBN–the amount of initiator with respect to the total number of reactive double bonds present in the system.

To characterize the polymers, 4 resultant *dependent variables* ($y_1$, $y_2$, $y_3$, $y_4$), also called objective parameters, were considered, which in our case are:

- $B_{max}$–the maximal absorption of the target analyte at high, saturating analyte concentration, which refers to the capacity (aim: increase for MIP, decrease for NIP).
- $K_{50}$–the polymer concentration at half maximum absorption of the analyte, an equilibrium constant, as a measure of the affinity (aim: decrease for MIP).
- $B_2$–the relative absorption of the target analyte bound to 2 mg/mL of polymer, at low analyte concentrations, as a measure for the performance of the MIP under conditions found in trace analysis (aim: increase for MIP, decrease for NIP).

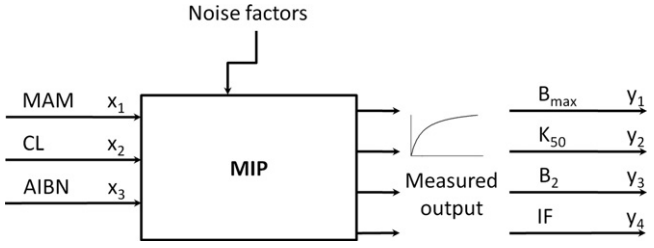


**Fig. 3.** Block diagram of influencing factors ($x_1-x_3$) as input and objective parameters (dependent variables $y_1-y_4$) as derived output parameters of the polymer system. MAM, CL, AIBN are influencing factors whereas $B_{max}$, $K_{50}$, $B_2$ and imprinting factor (IF) are binding parameters. As uncontrollable noise factors, environmental influences (e.g. temperature and weighing errors) may be considered.

- IF–imprinting factor (ratio of binding to the MIP/binding to the non-imprinted control polymer, NIP) at 2 mg/mL polymer concentration at low analyte concentration (aim: increase).

The 3 influencing factors considered here are each varied on two defined factor levels (Table 1). The height and consequently the distance of the factor levels were set such that the expected influence is not too small (not to be mixed with noise) and that at the same time the distance between the levels would not be too high, in order to remain in the linear range. To obtain effects that are above the noise level a theoretical error analysis based on the propagation of uncertainty was performed in order to judge the expected variations of each factor by pipetting and weighing errors. For this, the factor levels were specified to such an extent that they are at least three times bigger than the calculated uncertainty. Furthermore, the range of influential parameters was determined so that the general behavior of the polymer ("system") is stable in order to apply one mathematical model for the whole design space and not to split it into parts with different "system" properties. A stable polymer "system" behavior means that the state variables, for instance, physical softness and opalescence are constant on a macroscopic level.

Unless there is a wide theoretical range of variation of the influential factors within the above described limits, there are some more general limitations that confine the parameter range. Namely, an initiator concentration of 1 mol% or even lower is favorable as shown in [23] where an experimental design showed the dependency of the separation factor on the initiator concentration ranging from 1 to 5 mol%. The initiator concentration is calculated as the mole percentage of all double bonds able to participate in free-radical polymerization. The degree of cross-linking (CL, %) refers to the relative percentage of cross-linking monomer to all monomers by weight. The higher this degree the more rigid is the polymer. Finally, the molar ratio between functional co-monomer and template MAM is expressed as molar equivalents. The molar ratio of AAB to GA (1:1) and the total concentration of monomers ($c_m$ 5%) were kept constant. The composition of the starting point polymer, which corresponds to run 7 in the design is $c_m$ 5%, AIBN 0.56 mol%, CL 83%, 7 MAM:1 AAB:1 GA.

The plan and design space were designed in order to have unambiguous information about the impact of each factor and its interactions. Therefore a full factorial design with 3 factors and 2 factor levels that still has a manageable number of experiments was used. The experimental plan is shown in Table 2 with $2^3=8$ experiments and 3 center points (to determine whether there is a linear or non-linear relationship between the factors and the response).

Non-linear curve fitting to a Langmuir-type isotherm equation of the equilibrium binding isotherms obtained by radioligand binding experiments was performed in order to determine the values of the dependent variables ($K_{50}$, IF, $B_2$) referring to:

$$m_{GA} = m_{GAmax} \cdot m_{Pol}/(K_{50}+m_{Pol})$$

$m_{GA}$ refers to the amount of bound radioligand [$^{14}$C]GA to the polymer and $m_{Pol}$ refers to the polymer mass in mg, $K_{50}$ is the polymer concentration at half maximum absorption of the analyte and $m_{GAmax}$ refers to the maximal amount of bound [$^{14}$C]GA. The imprinting factor (IF) was determined by the ratio $m_{GA}$ of radioligand binding to the MIP/binding to the NIP at 2 mg polymer respectively. $B_2$ was determined by the amount of bound [$^{14}$C]GA at 2 mg polymer. $B_{max}$ was determined in a separate saturation experiment. The fitted parameters and all other dependent variables are represented in Table S1. All data were analyzed with the software Statistica 9.0 (StatSoft, Germany).

This comprises the ANOVA, regression analysis and multi-objective optimization.

To detect significant factors and their interactions an analysis of variance (ANOVA) was performed for each factor and its interactions with other factors. All possible interactions (up to 3-factor interactions) were considered. The significance level $\alpha$ for the p value was fixed to 5%.

The impact of the effects (responses) of the dependent variables was predicted by adjusting the observations to a model function by multivariate regression analysis of all significant factors. The data were fitted to a model by multiple linear regression. The second degree polynomial was applied as regression model, which is a linear combination of the main factors $x_i$ and all possible interactions $x_i x_j$ (up to 2-factor-interactions):

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 \quad (1)$$

whereas $y$ refers to the dependent variables, $b$ are weights with $b_0$ as a constant and $x_i$ are the independent variables MAM, CL, and AIBN (as shown in Fig. 3). The influencing factors $x_i$ were coded (normalized) to make all parameters dimensionless and to compare their impact directly. An extensive residues analysis and the check of correlations between predicted and measured values were done by scatter plots.

### 3.3. Effects of influencing composition factors on the MIP binding parameters of the polymer library

Equilibrium binding experiments with radiolabeled GA were performed with all polymers, keeping the amount of radioligand low and constant, and varying the polymer concentration. The results from curve fitting to a Langmuir-type isotherm equation ($B_2$, $K_{50}$, IF) are represented in Table S1 and Fig. S1 (Supplementary material). In addition, the maximum binding capacity at saturation ($B_{max}$) was also determined at high analyte concentration (100 μM glucuronic acid). All parameters were first analyzed with ANOVA and statistically significant influences of the independent variables on the dependent variables were modeled by a regression analysis to a linear model (Eq. (1)) in order to judge about the impact of each influencing factor on each of the respective dependent variables. An overview of all regression models with all significant factors is given in Table 3. The $R^2$ value and a residues analysis give both insights into the goodness of fit of the models to the data. As a result, the models fit the data well, since $R^2$ is with one exception higher than 69%, the residues lack a dependency on the order of the experiments, and the height of the predicted values and all residues are normally distributed. Concerning the parameter $R^2$ one has to keep in mind that the applied models are simplified linear combinations of the real mathematical description and by reducing the model by all insignificant factors. Furthermore as an example the comparison of predicted to obtained binding properties in methanol:water 9:1 of polymer 3 revealed that all obtained binding properties lie within the 95% interval of the predicted parameters (Table S3, Supplementary material).

Based on the regression analysis the following results were obtained. $K_{50}$ for the MIP can be decreased by reducing the molar ratio of MAM to template and reduce the degree of cross-linking whereas $K_{50}$ of the reference polymer can be increased by increasing the degree of cross-linking. Moreover, the impact of influence of CL on the reference polymer is higher than on MIP (higher value of the regression coefficient). Therefore to achieve a high difference between MIP and reference polymer concerning binding affinity (decreased $K_{50}$ of MIP, increased $K_{50}$ of NIP) the functional monomer ratio to GA should be decreased. At the same time the cross-linking degree could theoretically be increased for improvement as it has a stronger effect on NIP compared to MIP. Thus, it might be conflictive to fulfill all aims at the same time.

$B_{max}$ and $B_2$ of MIP can be increased by reducing the MAM to GA ratio and the degree of cross-linking. Furthermore there are two interactions of main factors on the height of $B_2$ of MIP which are: MAM to GA ratio and the degree of cross-linking (MAM · CL) as well as the degree of cross-linking and the initiator concentration (CL · AIBN) which are both highly significant. $B_2$ of the reference polymer can only be influenced by MAM · CL. These interactions make it difficult determine the optimal factor levels that optimize all objective parameters at the same time.

To sum up, a decreased MAM to GA ratio results in a decreased $K_{50}$ and increased $B_{max}$ and $B_2$ as desired. At the same time the MAM to GA ratio has no effect on NIP or just an effect that depends on the degree of cross-linking (MAM · CL interaction). The degree of cross-linking has an effect on all objective parameters of MIP and the reference polymer. Therefore its overall impact needs to be considered in detail (see chapter 3.4. multi-objective optimization).

The imprinting factor (IF) can not be actively influenced by any of the considered factors (no significant effects were determined). This might be a disadvantage for an optimization strategy but at the same time can be advantageous concerning the robustness of the imprinted polymer as a change of any of the parameters in the considered range will not dramatically change the polymer binding behavior (here: nonspecific binding).

The results show that the degree of cross-linking and functional monomer have the highest impact on the binding properties which is in agreement with previously published work of Navarro-Villoslada et al. [16] where the amount of cross-linker and functional monomer were the most influential factors whereas the amount of template and initiator, the volume of the porogen and the initiation procedure (UV, thermal) just had a minimal effect on the affinity. In our case, the nonspecific binding properties of the reference polymer are dependent on the degree of cross-linking. At the same time the specific binding properties of the MIP were always dependent on the functional monomer.

There is a significant influence of at least one factor on each dependent variable shown in Table 3, which implies that the experimental design can potentially improve the characteristics of the polymers. The parameters of the regression model give insights into the impact (height and direction) of all factors affecting each of the dependent variables. As these relations are now well known one might wish to use this information to optimize all objective parameters at the same time, but as shown before there might be conflictive aims where compromises have to be made. Moreover the interaction of factors make it difficult determine the optimal factor levels that optimize all objective parameters. A multi-objective optimization analysis might help to overcome this problem and to find a polymer with optimal

**Table 3**
Data obtained by the regression analysis for polymer binding properties in methanol:water 9:1 with model coefficients $b_0 - b_n$ for each of the considered dependent variables $y$ (M $K_{50}$, R $K_{50}$, M $B_{max}$, M $B_2$, R $B_2$, M refers to imprinted and R to reference polymer) for the polynomial model (Eq. (1)). The constant refers to $b_0$, and the other model parameters $b_1$–$b_n$ refer to each of the indicated normalized independent variables x and their interactions. E.g. the model for M $K_{50}$ is the following: M $K_{50} = 0.103 + 0.0072 \cdot \text{MAM} + 0.139 \cdot \text{CL}$; $R^2$–coefficient of determination.

| Factor | M $K_{50}$ (mg/mL) | R $K_{50}$ (mg/mL) | M $B_{max}$ (μmol/mg) | M $B_2$ (pmol) | R $B_2$ (pmol) |
|---|---|---|---|---|---|
| Constant | 0.103 | 0.145 | 0.0206 | 13.2 | 10.0 |
| MAM | 0.072 | | −0.0033 | −0.9 | |
| CL | 0.139 | 0.231 | −0.0064 | −1.3 | |
| MAM · CL | | | | −1.1 | −1.9 |
| CL · AIBN | | | | −1.1 | |
| $R^2$ | 0.77 | 0.69 | 0.86 | 0.92 | 0.41 |

binding properties within the design space which combines all objectives as a compromise.

### 3.4. Multi-objective optimization

To determine the factor levels of the independent variables (component concentrations) that optimize the effect on multiple dependent variables (binding properties of the polymers) a multi-objective optimization was performed. The applied desirability approach of Derringer and Suich [28] is based on the assignment of "desirability values" to the dependent variables in such a way that the most desired (optimal) value from the range of possible values achieved by the experimental plan gets the desirability value 1 and the most undesired value the desirability value 0. With this method, not just the best polymer out of a repertoire of synthesized polymers can be determined but even a prediction about a polymer composition with globally best binding properties may be obtained. The general idea is to optimize the MIP by deciding which dependent variables should be optimized in which manner (maximized, minimized or optimum in the middle range) by using desirability functions. The desirability functions for each of the objective parameters are all linear implying the more the better or the less the better. For example, the $K_{50}$ of the MIP should be minimized, therefore the minimal $K_{50}$ value gets the desirability value of 1.

Within the obtained desirability functions the independent parameter combination that maximizes the overall desirability is determined by a univariate search function. The overall desirability is the geometric mean of all desirability functions. With these optimal independent parameters the response function is created, which can be presented as surface response plot (Fig. 4). For this approach a global regression model was used containing all independent variables that have a significant effect on at least one of the dependent variables. In Fig. 4 it can be seen that a low MAM to GA ratio and a high degree of cross-linking optimizes the desirability in the surface response plot (Fig. 4A). Furthermore, when the cross-linking is high the initiator should be medium concentrated in order to optimize the overall MIP properties as it can be seen in plot Fig. 4C. As a low MAM to GA ratio was optimal, the initiator concentration should be around 0.65% to optimize the desirability (Fig. 4B). The calculated values for the ideal MIP within the design space are the following (model with just significant independent variables for at least one dependent variable $M\ K_{50}$, $R\ K_{50}$, $M\ B_{max}$, $M\ B_2$, $R\ B_2$): 83% degree of cross-linking; 3 eq of MAM and 0.65 mol% of initiator AIBN.

The optimal ratio of template:monomers:cross-linker is GA:(AAB+MAM):EDMA is 1:(1+3):20 (GA:AAB was maintained fixed at 1:1). This somehow confirms the predominant opinion from the literature, where ratios of template:functional monomers:cross-linker of 1:4:20 are most often used [24,29,30]. In our example, the design of experiments approach provides the basis for a global MIP optimization within the design space.

From the overall desirability the theoretically expected optimal polymer composition can be computed depending on the objective of the experimenter (which binding parameters and their weighing). Therefore, it is an appropriate tool to tailor a MIP to a specific application.

The applied multi-objective optimization method based on the desirability approach of Derringer and Suich [28] needs low computational expenses due to the applicability of standard optimization engines used for univariate optimization. Therefore, this method is, in contrast to other multi-objective optimization methods that rely on *a posteriori* articulation of preferences of the decision-maker, like genetic algorithms, very easily applicable, computationally straightforward and fast [31]. Furthermore this method entails just one solution — one sole optimal MIP composition — and not a Pareto optimal set of solutions [32]. The latter is not desired in the context of extensive efforts for MIP synthesis and characterization. Compared to other methods with *a priori* articulation of preferences, the use of the geometric mean to determine the overall desirability function is less subjective compared to the weighted sum method. In summary the choice of the appropriate multi-objective optimization method depends on (a) when the preferences of the decision-maker should be articulated: before (*a priori*) or after (*a posteriori*) having a Pareto optimal set of solutions, (b) the computational efforts and (c) the application (e.g. concerning the amount of objective functions).

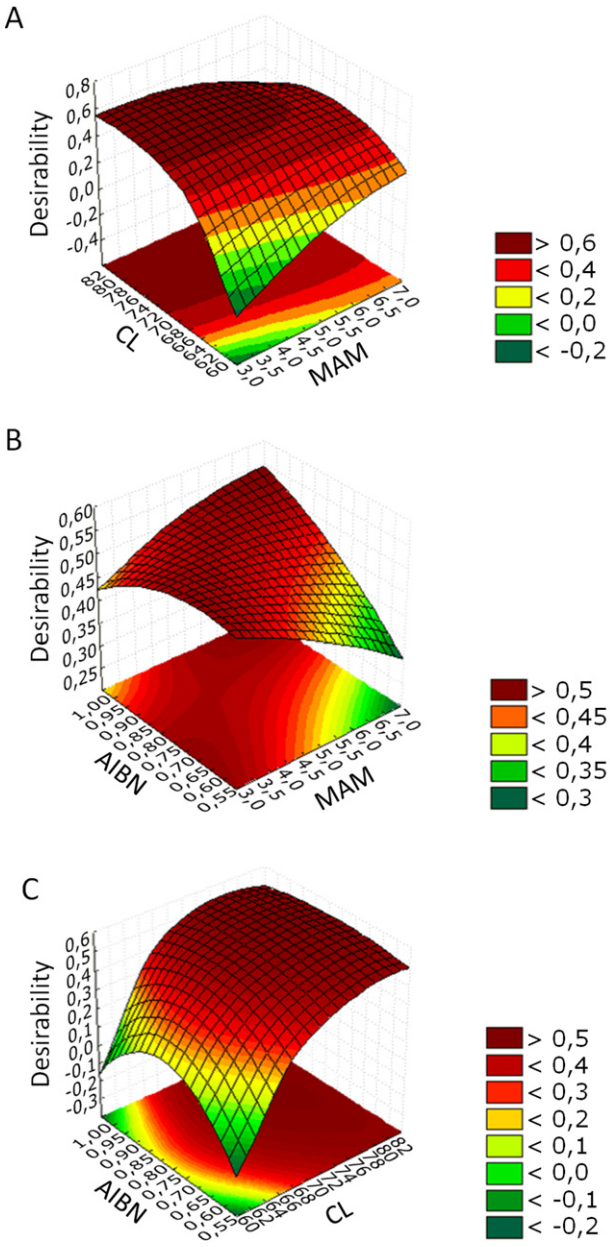


**Fig. 4.** Surface response plots for all parameter combinations. Higher desirability values correspond to an optimal parameter combination. (A) Degree of cross-linking (CL) versus MAM to GA ratio, (B) initiator AIBN concentration versus MAM to GA ratio, and (C) AIBN versus CL.

### 3.5. Improved binding properties of the new designed imprinted polymer

The theoretical optimal polymer (no 10) obtained by multi-objective optimization was synthesized and characterized (Fig. 5A).

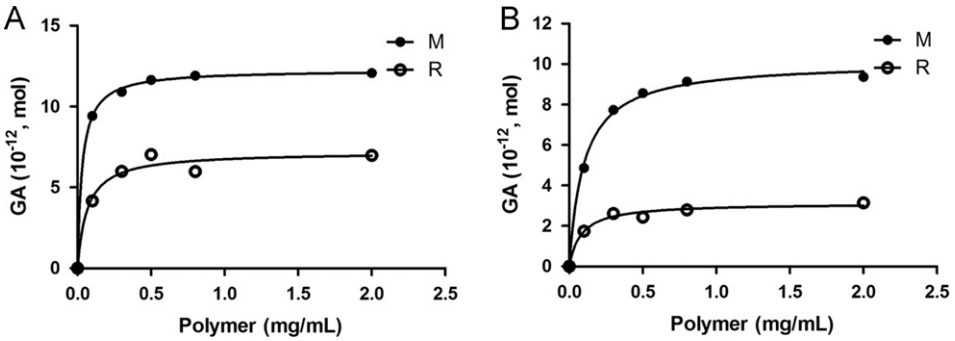


**Fig. 5.** (A) Equilibrium binding isotherms of polymer no 10 in methanol:water 9:1; M — imprinted polymer, R — reference (non-imprinted) polymer. (B) Equilibrium binding isotherms of polymer no 3 in pure water; M — imprinted polymer, R — reference (non-imprinted) polymer.

**Table 4**
Data obtained by the regression analysis for polymer binding properties in water with model coefficients $b_0-b_n$ for each of the considered dependent variables $y$ (M $K_{50}$, R $K_{50}$, M $B_2$, R $B_2$, IF, M refers to imprinted and R to reference polymer for the polynomial model (Eq. (1)). The constant refers to $b_0$, and the other model parameters $b_1-b_n$ refer to each of the indicated normalized independent variables $x$ and their interactions. E.g. the model for M $K_{50}$ is the following: M $K_{50}=0.080+0.074\cdot$CL; $R^2$ — coefficient of determination.

| Factor | M $K_{50}$ (mg/mL) | R $K_{50}$ (mg/mL) | M $B_2$ (pmol) | R $B_2$ (pmol) | IF |
|---|---|---|---|---|---|
| Constant | 0.080 | 0.084 | 10.3 | 7.9 | 1.478 |
| MAM | | | −2.0 | | |
| CL | 0.074 | 0.071 | −3.5 | −4.8 | 0.773 |
| AIBN | | 0.029 | | 2.4 | −0.922 |
| CL · AIBN | | 0.032 | | | −0.651 |
| $R^2$ | 0.62 | 0.88 | 0.75 | 0.82 | 0.84 |

The improvement of binding properties in comparison to the starting point polymer before optimization by DoE with multi-objective optimization is the following: 6.2 times decreased $K_{50}$ of MIP, which corresponds to a higher affinity, 40% higher capacity ($B_{max}$) and 1.3 times increased IF. $B_2$ itself stayed constant for MIP, but for NIP it was decreased by about 17%. The dissociation constant $K_d$ of this best polymer no 10 was determined by 3 independent measurements to be 17.3 μM and its capacity is 18 mg/g in methanol:water 9:1. These results illustrate the power of experimental design coupled with multi-objective optimization to find a global optimal polymer composition within the design space with improved MIP binding properties.

### 3.6. Analyte recognition in aqueous media

In order to evaluate the possibility to apply the GA-imprinted polymers to biological samples, binding assays of all polymers were also performed in water (Table S2 and Fig. S3, Supplementary material).

The obtained binding parameters were analyzed in the same manner with ANOVA and regression analysis (Table 4). The model fits the data well, which is reflected by $R^2$ above 62% and by the residues analysis. Furthermore as an example the comparison of predicted to obtained binding properties in pure water of polymer 3 revealed that all obtained binding properties lie within the 95% interval of the predicted parameters (Table S4, Supplementary material).

From this binding data in water it is obvious that the binding properties are changed with respect to binding in methanol:-water 9:1, but not dramatically. For instance, the effect of the degree of cross-linking stayed high for all objective parameters. At the same time the impact of the initiator is increased and the general impact of MAM is decreased in water compared to the methanolic environment. The latter is probably due to the fact that hydrogen bonding to MAM plays a lesser role due to the stronger competition by water molecules. It is informative that the height of the imprinting factor is significantly influenced by several factors (degree of cross-linking, initiator, interaction between the degree of cross-linking and the initiator concentration (CL · AIBN)), which was not the case in the water-methanol mixture. Therefore it seems that the MIP binding properties can be tailored even better in water. The polymer with the best binding properties concerning all considered binding parameters in water was no 3 instead of no 10 which was the best in methanol:water 9:1.

To show the changing binding properties in pure water compared to a methanolic environment different fractions of water (10, 30, 50, 80, 100 v/v%) were added to the incubation solvent methanol of polymer no 3. The equilibrium binding isotherms are shown in Fig. S2 (Supplementary material). As it can be seen, increasing the fraction of water in the incubation solution, the non-specific binding is decreasing together with $K_{50}$. Besides, the highest imprinting factor (IF=3.2) was achieved in pure water (Fig. 5B). This result illustrates that molecular recognition can be tuned in several solvents as at least two binding parameters are dramatically changed dependent on the fraction of water.

## 4. Conclusions

To the best of our knowledge, it is the first time that a comprehensive multi-objective optimization of the binding properties of a MIP has been performed, which was applied to the non-covalent molecular imprinting of a polar target, glucuronic acid. With the design of experiments approach coupled with a multi-objective optimization method not just the best polymer out of a repertoire of synthesized polymers can be determined but even a prediction about a polymer composition with global best binding properties could be obtained. Here, an optimal glucuronic acid binding polymer composition was found with 0.65 mol% of initiator and 1:3:20 ratio of template:co-functional monomer:cross-linker (T:M:X), plus 1 equivalent of the stoichiometric monomer AAB. With this optimization method the imprinted polymer could be improved compared to the original starting polymer with approximately 6.2 times lower $K_{50}$ which corresponds to a higher affinity, 20% higher capacity at low analyte concentration ($B_2$), 40% higher capacity ($B_{max}$) and 1.3 times increased imprinting factor at 2 mg polymer concentration.

In pure water the polymers in some cases better binding than in methanol:water 9:1 was obtained. We showed that the applied multi-objective approach coupled with DoE can be used to tailor the properties of an imprinted polymer to a specific application.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2012.11.029.

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