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Fluorescent water soluble polymers for isozyme-selective interactions with matrix metalloproteinase-9

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ABSTRACT

Matrix metalloproteinases (MMPs) are overexpressed in various pathological conditions, including cancers. Although these isozymes have similar active sites, the patterns of exposed amino acids on their surfaces are different. Herein, we report the synthesis and molecular interactions of two water soluble, fluorescent polymers which demonstrate selective interactions with MMP-9 compared to MMP-7 and -10.

Water soluble, flexible polymers have been used by various groups to selectively bind to different proteins.[1–3] The flexibility of the polymer backbone aids in the formation of multiple, complementary interaction sites between the polymer and the amino acid residues on the protein surface. These polymers have been demonstrated to be useful as affinity membranes, as switches to 'turn on' enzyme activity, as selective protein immobilization agents, as protein sensors, etc.[1–3] However, the proteins used in these selective recognition experiments are structurally very different (e.g., lysozyme, bovine serum albumin, cytochrome c, etc.). To the best of our knowledge, selective binding of polymers to different isozymes of an enzyme family has not been demonstrated.

Matrix metalloproteinases (MMPs) are a group of Zn^{2+} containing metalloenzymes capable of hydrolyzing the extracellular matrix.[4,5] These enzymes are involved in a number of different physiological processes, for example, cell proliferation, apoptosis, differentiation, angiogenesis, chemokine/cytokine activation and the expression levels of these enzymes are strictly regulated at multiple levels.[6–9] Various MMP isozymes (in particular MMP-9) have been shown to be upregulated in degenerative diseases, for example, arthritis, multiple sclerosis, metastatic cancers, etc.[9–11] In healthy individuals, the serum concentration of MMP-9 is about 5–10 nM.[12] For lung cancer patients, the concentration of this enzyme can be as high as 100–200 nM in the bronchial lavage fluids.[13] The levels of MMP-9 serve as diagnostic and prognostic markers for these diseases.[12,13]

Since isozymes catalyze the same chemical reaction, their active sites are remarkably similar.[14] However, the pattern of amino acid residues on the surface of the isozymes are not under evolutionary pressure and are usually not conserved.[15] We reasoned that this difference can be exploited for selective binding of polymers to one isozyme in preference to the others. Herein, we report our results on selective binding to recombinant human matrix metalloproteinase-9 (MMP-9) by a set of water soluble, flexible polymers.

We synthesized two sets of monomers using two different polymerizable moieties. The first set included the use of a commonly used water soluble compound methacrylic acid. For the second set, we choose to examine the effects of a benzene ring incorporated into the structure and 4-vinylbenzoic acid was used. We conjugated the polymerizable moieties to an alcohol (for increased water solubility and hydrogen bonding with amino acid residues on the surface of MMPs), to the fluorogenic dansyl group and to a non-selective MMP inhibitor. Different amino acids such as lysine (positively charged), aspartic acid (negatively charged) and β-alanine (non-polar) were also linked to the polymerizable groups to impart electrostatic and hydrophobic properties to the polymers (Scheme 1, synthetic details are provided in Supplementary data).

Random copolymers were prepared from these synthesized monomers using azoisobutyronitrile (AIBN) as the free-radical

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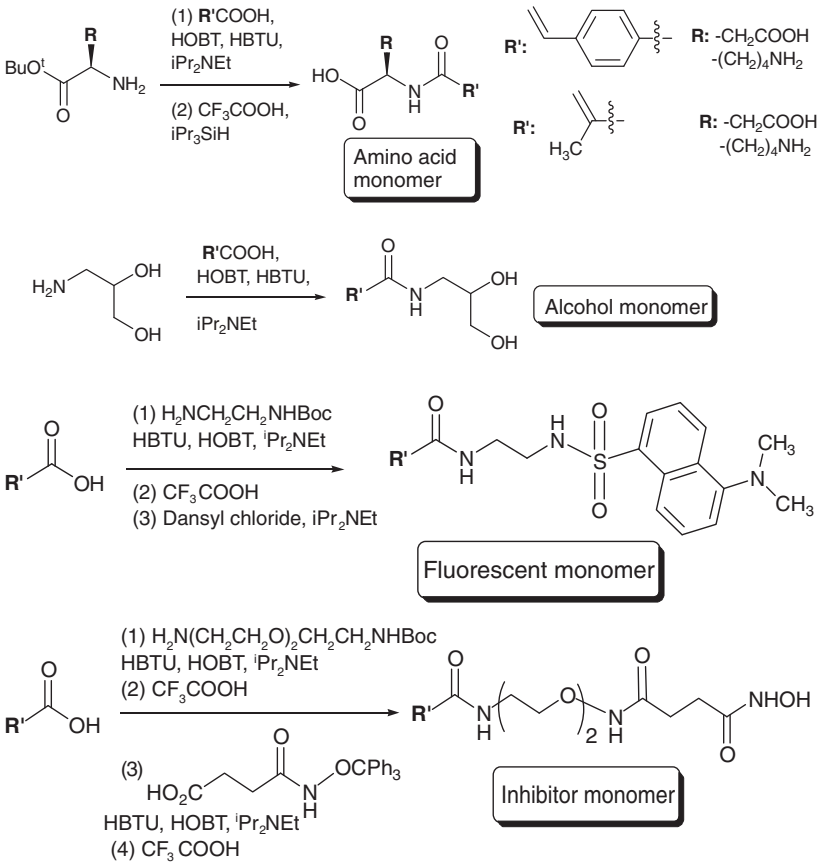

Scheme 1. Synthesis of methacrylamide and 4-vinylbenzamide based monomers.

initiator following a literature protocol.[2,3] Because of flexibility, we decided to prepare a small set of polymers and screen for selective interactions with MMP-9. We systemically varied the starting monomer compositions (Table 1) and studied the interactions of the resultant polymers with recombinant human MMP-7, -9 and -10. The molecular weights of the polymers were determined by gel permeation chromatography (GPC). The molecular weights were in the range of 83–129 kDa with polydispersities in the range 1.2–2.5 (Table 2).

Table 1
The starting monomer amounts (mol %) for the synthesized methacrylamide ($\mathbf{R^1}$–$\mathbf{R^{11}}$) and 4-vinylbenzamide-based polymers ($\mathbf{M^1}$–$\mathbf{M^7}$)

Polymer	Monomers (mol %)					
	Dansyl	Alcohol	Inhibitor	Asp	Lys	Ala
$\mathbf{R^1}$	10	80	10	—	—	—
$\mathbf{R^2}$	10	90	—	—	—	—
$\mathbf{R^3}$	10	80	—	—	10	—
$\mathbf{R^4}$	10	80	—	10	—	—
$\mathbf{R^5}$	10	70	—	10	10	—
$\mathbf{R^6}$	10	60	10	10	10	—
$\mathbf{R^7}$	10	70	10	10	—	—
$\mathbf{R^8}$	10	70	10	—	10	—
$\mathbf{R^9}$	10	80	—	—	—	10
$\mathbf{R^{10}}$	10	70	10	—	—	10
$\mathbf{R^{11}}$	10	50	20	10	10	—
$\mathbf{M^1}$	10	80	—	—	10	—
$\mathbf{M^2}$	10	70	10	—	10	—
$\mathbf{M^3}$	10	80	—	10	—	—
$\mathbf{M^4}$	10	70	10	10	—	—
$\mathbf{M^5}$	18	45	19	9	9	—
$\mathbf{M^6}$	12	64	—	12	12	—
$\mathbf{M^7}$	11	78	—	—	—	11

Table 2
The weight average (M_w) and number average (M_n) molecular weights, polydispersities (P.I.) of the polymers $\mathbf{R^1}$–$\mathbf{R^{11}}$, $\mathbf{M^1}$–$\mathbf{M^7}$ and the concentrations used during the titration experiments with MMP isozymes

Polymers	M_w	M_n	P.I.	Concn used (nM)
$\mathbf{R^1}$	128,920	66,623	1.93	27
$\mathbf{R^2}$	107,291	43,945	2.44	34
$\mathbf{R^3}$	99,486	50,436	1.97	31
$\mathbf{R^4}$	119,800	52,727	2.27	28
$\mathbf{R^5}$	96,232	37,139	2.59	32
$\mathbf{R^6}$	94,514	52,959	1.78	34
$\mathbf{R^7}$	116,450	47,670	2.44	26
$\mathbf{R^8}$	93,405	47,258	1.98	36
$\mathbf{R^9}$	117,010	76,187	1.54	27
$\mathbf{R^{10}}$	106,802	67,273	1.59	30
$\mathbf{R^{11}}$	117,191	64,577	1.81	31
$\mathbf{M^1}$	106,622	66,942	1.59	29
$\mathbf{M^2}$	128,790	104,510	1.23	24
$\mathbf{M^3}$	83,618	40,895	2.04	36
$\mathbf{M^4}$	115,498	72,963	1.58	26
$\mathbf{M^5}$	114,428	78,161	1.46	27
$\mathbf{M^6}$	89,801	37,978	2.36	34
$\mathbf{M^7}$	127,656	77,145	1.65	24

To study the interactions of the synthesized polymers with the MMP isozymes, nanomolar solutions of the polymers (25–35 nM in 30 mM phosphate buffer, pH 7.4, Table 1) were prepared and the MMP isozymes were added to make 200 nM of the final enzyme concentration (details are provided in Supplementary data). The changes in the emission spectra of the polymer-incorporated dansyl group were recorded in the region 350–750 nm (λ_{ex} = 325 nm; Fig. 1 and Supplementary data). The emission intensity was found to decrease in the presence of added enzymes. This possibly reflects that the polymer-incorporated dansyl groups are experi-

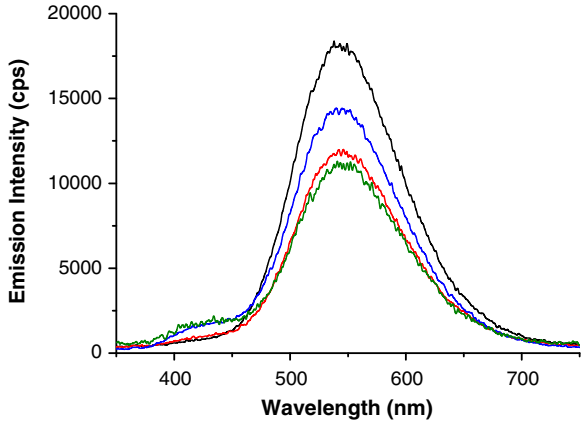

Figure 1. Fluorescence emission spectra of the polymer $\mathbf{R^{11}}$ (31 nM in 30 mM phosphate buffer, pH 7.4, λ_{ex} = 325 nm, black trace) in presence of 200 nM of recombinant human MMP-7 (red trace), MMP-9 (blue trace) and MMP-10 (green trace).

encing more hydrophilic microenvironments in the presence of recombinant enzymes.[20] We observed that the relative decrease in emission intensity (541 nm for polymethacrylamide polymers $\mathbf{R^1}$–$\mathbf{R^{11}}$ and 510 nm for polyvinylbenzamide polymers $\mathbf{M^1}$–$\mathbf{M^7}$) depends on the polymer used and the MMP isozyme tested. The ratios of the fluorescence emission intensities (at 541 nm and 510 nm) in the absence and in the presence of the MMP isozymes were calculated and subjected to statistical analyzes to determine if they are significantly different.

The primary objective of the statistical analysis was to determine whether (and to what extent) each of the polymer selectively interacts with MMP-9 isozyme. Because each of the random copolymer groups (methacrylamide and 4-vinylbenzamide, see Table 1) is fundamentally different in structure, a decision was made to analyze each group separately. A common approach to identify these relationships is linear discriminant analysis.[16,17] However, the large number of polymers (11 in the methacrylamide random copolymer group and 7 in the 4-vinylbenzamide group, including one control for each group) relative to the small number of replications (6) for each enzyme–MMP pair made this approach infeasible.[18] A commonly used alternative, employed in this analysis, is (binary) logistic regression.[19] More specifically, each of the 18×1 data matrices containing the fluorescence intensities for a given polymer (6 replications and 3 MMPs per polymer) were 'stacked' or 'blocked' together to form a larger (198×1 for the methacrylamide random copolymer group and 144×1 in the 4-vinylbenzamide group) vector of fluorescence intensity readings (11 polymers with 18 observations per polymer for the methacrylamide group and 7 polymers with 18 observations for the 4-vinylbenzamide group, respectively).[19] Additional columns in the data matrix were appended by creating a series of binary variables that identify each polymer, and these binary indicator variables were subsequently interacted with the fluorescence data to create a fully interacted data matrix (198×22 and 144×16 for each copolymer group, respectively). Lastly, three columns of binary variables were appended, where each new column identified a particular MMP (-7, -9 or -10). The full data matrix are provided in the Supplementary data accompanying this manuscript.

The value of this approach is that it allows for the estimation of a fully interacted logit model of the following form:

$$P(\mathrm{MMP}_i^k = 1 \mid D, F) = \sum_{j=1}^{J} D_i^j(\beta_j + \gamma_j F_i^j)$$

$$P(\mathrm{MMP}_i^k = 0 \mid D, F) = 1 - P(\mathrm{MMP}_i^k = 1 \mid D, F) \qquad (1)$$

where i indexes each observation; $P()$ denotes the cumulative logistic distribution; D is a binary indicator of each (j = 1,...,J) polymer, k denotes each isozyme (MMP-7, -9, and -10), F is the fluorescence of each isozyme; J denotes the number of polymers (11 and 7, respectively) and β, γ are parameter estimates. While this is estimated as single maximum likelihood estimation, the stacked data matrix allows separate intercept and slope estimates for each polymer included in the regression. That is, each of the polymer-specific response functions in a given group is 'stacked' and estimated together to preserve adequate degrees of freedom to run the regression. As noted earlier, Eq. (1) is estimate twice, once for each random copolymer group.

Because the goal of the analysis was to indentify which polymers selectively interacts with MMP-9, Eq. (1) was estimated as a binary logit model, where the dependent variable takes a value of one for a MMP-9 isozyme, and zero if the isozyme is in the remaining categories (MMP-7, -10). This ensures a parsimonious estimation procedure without being forced to estimate Eq. (1) multiple times for each isozyme-copolymer grouping (MMP-9 vs MMP-7, MMP-9 vs MMP-10, etc.). Additionally, because each polymer has a separate response function with two parameter estimates (a slope and an intercept), results are presented using an odds ratio (with accompanying 95% profile confidence intervals) capturing the joint effects of these intercept and slope parameters for a given polymer on the MMP-9 isozyme. Values greater than unity (and whose confidence intervals do not contain a value of one) indicate that the polymer significantly predicts or identifies the given MMP relative to its alternatives.

Table 3 identifies those polymers whose odds ratios are significantly greater than unity. This statistical analysis revealed that it is possible to design polymers that are able to selectively interact with isozymes within the MMP family. In particular, note that polymer $\mathbf{R^{11}}$ uniquely and significantly interacts with the MMP-9 isozyme in the methacrylamide copolymer group. Polymer $\mathbf{M^5}$ uniquely and significantly interacts with MMP-9 among the 4-vinylbenzamide copolymer group.

Both of $\mathbf{R^{11}}$ and $\mathbf{M^5}$ polymers contain the aspartic- and the lysine monomers (10 mol % each) and 20 mol % of the inhibitor monomer. These two polymers also contain the least amounts of the alcohol monomers. Decreasing the amount of the inhibitor monomer to 10 mol % (i.e., polymers $\mathbf{R^6}$), omitting any charged monomers (polymers $\mathbf{R^8}$, $\mathbf{M^2}$ and $\mathbf{M^4}$) or the inhibitor monomer from the polymers (i.e., $\mathbf{R^5}$, $\mathbf{M^6}$) led to the loss of selective interactions of the polymers with MMP-9 (Supplementary data, Table S3). Reducing the amount of inhibitor monomer (to 10 mol %) (i.e., polymer $\mathbf{R^6}$) or incorporation of more hydrophobic alanine-based monomer in the polymer (i.e., $\mathbf{R^{10}}$ and $\mathbf{M^7}$) also had negative effect on the selective interactions with MMP-9 (Supplementary data, Table S3). We also observed that the polymers $\mathbf{R^{11}}$ and $\mathbf{M^5}$ (100 nM each) are effective in inhibiting the activity of the enzyme MMP-9 (Supplementary data). Although we did not systematically

Table 3
Estimates and p-values from the logit regression analyzes

	Methacrylamide-based polymer	4-Vinylbenzamide-based polymer
Polymers	$\mathbf{R^{11}}$	$\mathbf{M^5}$
Intercept	43.644	101.8
Intercept p-value	0.017	0.025
Slope	−32.235	−85.875
Slope p-value	0.016	0.026
Odds Ratio (OR)	187.598	>999.999
95% OR lower confidence interval limit	1.347	15.387
95% OR upper confidence interval limit	>999.999	>999.999

vary the steric effects in the monomers, these observations suggest that possibly the inhibitor on the polymers is interacting with the active site of MMP-9 and the charged amino acids are forming additional interactions with the amino acid residues on the surface of the enzyme. Incorporation of hydrophobic monomers was detrimental to the selectivity of interactions with MMP-9. However, increasing the amount of inhibitor monomer to 30 mol % in the polymers did not improve the selective binding to MMP-9 (data not shown). We do not have an explanation for this observation yet.

Next we proceeded to determine if the selective interactions of the polymers $\mathbf{R^{11}}$ and $\mathbf{M^5}$ are maintained in a complex mixture of proteins. The fluorescence emission from the polymer-incorporated dansyl group (λ_{ex} = 325 nm) was found to increase and blue-shift (about 100 nm; Fig. 2) in the presence of dilute (less than 5% by volume) human serum in phosphate buffer (pH 7.4). When this dilute human serum contained 200 nM of MMP-9 (levels of this enzyme in bronchial lavage fluid from lung cancer patients[13]), the emission intensity was substantially increased and blue-shifted (Fig. 2, blue trace). The same trends were observed when the human serum contained either 200 nM MMP-7 (Fig. 2, olive trace) or 200 nM MMP-10 (Fig. 2, magenta trace). This indicates that the polymer-incorporated dansyl fluorophore was experiencing a more hydrophobic microenvironment in the presence of dilute human serum and the MMPs.[20] We note that with the recombinant MMPs in buffer, the reverse was observed, that is, the emission intensities decreased in the presence of added enzymes. This may indicate that the conformations of the polymers change substantially when the buffer contains small amounts of human serum. Clearly, the emission intensity in the presence of MMP-9 was more pronounced in the presence of MMP-9 compared to MMP-7 and -10. Increasing the amount of human serum to more than 5% (by volume) led to the loss of the selective enhancements of the polymer emission intensity in the presence of MMP-9 (data not shown).

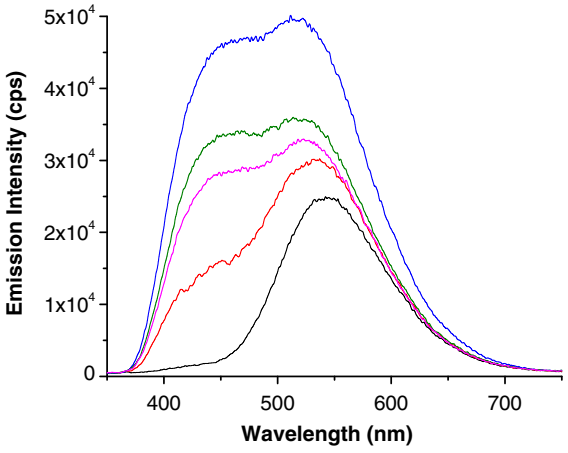

Figure 2. The fluorescence emission spectra of polymer $\mathbf{R^{11}}$ (50 nM) in phosphate buffer (30 mM, pH 7.4, λ_{ex} = 325 nm; black trace) was found to increase and blue-shift in the presence of dilute (2% by volume) human serum (red trace). The emission intensity changes were more pronounced when the dilute serum contained 200 nM MMP-9 (blue trace) compared to 200 nM MMP-7 (olive trace) or 200 nM MMP-10 (magenta trace).

In conclusion, we have synthesized flexible, water soluble polymers containing charges and a weak inhibitor for the MMPs. Two of these polymers demonstrated selective interactions to the isozyme MMP-9 compared to MMP-7 and -10, even in a complex mixture of proteins (e.g., dilute human serum). We anticipate that by incorporating more potent and selective MMP inhibitors in the polymer and by optimizing the polymer structures, the selectivity of the interactions with MMP-9 can be further enhanced and maintained in more concentrated human serum samples. These studies are currently in progress and the results will be reported in the future.

Acknowledgments

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Supplementary data

Supplementary (experimental details of the syntheses of the monomers and the polymers; details of the fluorescence experiments and detailed results of the statistical analysis) data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.02.020.

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