Lecture Notes in Bioengineering

Stefano Piotto
Simona Concilio
Lucia Sessa
Federico Rossi *Editors*

Advances in Bionanomaterials II

Selected Papers from the 3rd International Conference on Bio and Nanomaterials, BIONAM 2019, September 29 – October 3, 2019

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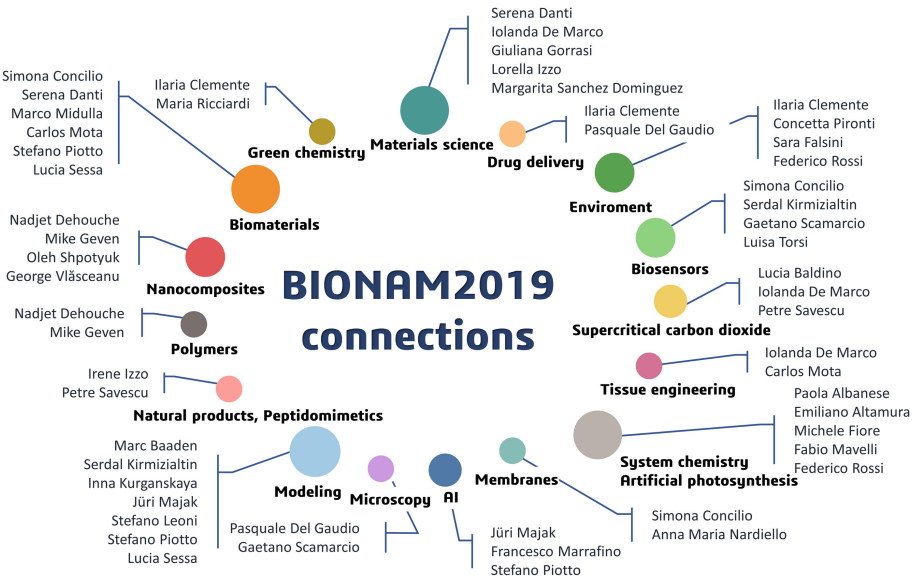
Preface

This volume of the Springer book series Lecture Notes in Bioengineering gathers the proceedings of BIONAM 2019, the 3rd Workshop on Bio-nanomaterials, held on September 29 – October 3, 2019, on an MSC cruise ship navigating the Mediterranean Sea. BIONAM focused on the analysis, synthesis and design of bionanomaterials. The previous editions were held in Salerno, Italy, in 2013 and 2016, respectively. The second edition was published by Springer with the title "Advances in Bionanomaterials."

Starting from well-known biological structures, scientists and engineers have developed design principles for novel nanomaterials with superior properties. This knowledge has permitted to create complex structures with high toughness and high mechanical strength, or having a remarkable variety of advanced properties.

The peculiar location where BIONAM 2019 took place allowed an intense interchange of ideas and favored cross-contamination among different fields. The workshop represented an effort to bring together researchers active in biomaterials modeling with experimentalists, and the connection map built out of the conference shows the network and the interactions developed among the different attendees.



Biophysicists, biochemists and bioengineers presented studies on the fundamental properties of materials suitable for medical use (e.g., implantable devices), for interaction with biological systems and environmental applications. Attendees from therapeutic areas highlighted essential features for developing compatible biomaterials and for the evaluation of such materials in a physiological environment. The computational scientists shared tools to predict the mechanical, physical or biological properties of new biomaterials.

As a whole, the conference provided a comprehensive yet not exhaustive picture of state of the art in the field of bio-nanomaterials. Compared to the previous editions, the 3rd conference gave emphasis to stimuli-responsive and adaptable smart materials. Several researchers coming from nonlinear and far-from-equilibrium chemistry were involved in the scientific committee to cover the new and hot discipline known as dynamical self-assembly, a branch of the material science that try to blend the classical equilibrium self-assembly theory with evolutionary chemical systems and dissipative structures.

As Editors, we wish to express gratitude to the organizing and secretariat committee from the University of Salerno, namely **Iolanda De Marco, Anna Maria Nardiello, Luigi De Biasi** and **Ylenia Miele**, and, of course, to all the attendees of the conference and to the authors who spent time and effort to contribute to this volume. We also acknowledge the precious work of the reviewers and the members of the Program Committee. Special thanks, finally, to the invited speakers for their highly inspiring talks: **Luisa Torsi** from the University of Bari (Italy), **Stefano Leoni** from Cardiff University (UK) and **Marco Midulla** from the University of Bourgogne (France).

The 21 papers presented have been thoroughly reviewed and selected from 54 submissions. The contributions are typical examples of research outcomes in the biomaterial area, and they were grouped into three main categories: “Structure and Properties of Bio and Nanomaterials,” “Modeling of Bio and Nanomaterials” and “Applications of Bio and Nanomaterials.”

They cover the following topics: *nanomaterials, smart and stimuli-responsive materials, applications of nanostructured materials to medicine and biology, supercritical fluids, nanovectors, modeling and simulation of artificial and biological systems, topical controlled release, complex systems, synthetic and systems biology, systems chemistry*, and they represent the most exciting contributions to the 2019 edition of BIONAM.

October 2019

Stefano Piotto
Simona Concilio
Lucia Sessa
Federico Rossi

Organization

BIONAM 2019 has been jointly organized by the University of Salerno (Italy) and the University of Siena (Italy) and held on a cruise in the Mediterranean Sea from September 29 to October 3, 2019.

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Structure and Properties of Bio and Nanomaterials

Composition and Microstructure of Biocompatible and pH-Sensitive Copolymers Prepared by a Free Solvent ARGET ATRP

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Abstract. Controlled/living radical polymerizations enable the synthesis of functional polymers with well-defined compositions and architectures. In this paper, we propose the usage of the ARGET ATRP technique to produce copolymers of MMA and DMAEMA. The feed composition was changed systematically to modulate the final composition of the copolymer. The absence of additional organic solvent (bulk polymerization), the use of low amounts of metal catalyst and the reduction of purification steps are the main advantages with respect to a traditional ATRP.

1 Introduction

Stimuli-responsive materials alter their chemical and/or physical properties upon exposure to external stimuli like pH, temperature, redox variations and light [1–5]. Nowadays, engineering of new responsive materials, especially block-copolymers, is a big scientific challenge and involve many researchers in finding new properties for applications that span from environmental monitoring and remediation to biological and medical applications [6–10].

A biologically relevant pH-responsive polymer is poly[2-(dimethylamino)ethyl methacrylate] (DMAEMA), which is both temperature- (lower critical solution temperature around 40 °C) [11] and pH-sensitive (the p*K*a of the amine group in poly-DMAEMA is around 7.3) [12]. Moreover, poly-DMAEMA is able to bind plasmid DNA through electrostatic interactions, yielding polymer/plasmid complexes (also called polyplexes) [13]. As observed for many polycations (see e.g. [14]), poly-DMAEMA is cytotoxic, but its cytotoxicity and hemolycity are reduced when DMAEMA is copolymerized with non-charged comonomers [15] (e.g. MMA). This strategy has been exploited to produce non-hemolytic bactericidal materials [16, 17].

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Indeed, linear and branched copolymers of MMA and DMAEMA (molar fraction = 25%) with mPEG segments (mPEG-*b*-poly(MMA-*ran*-DMAEMA)) gave not cytotoxic vesicles in the range concentration 10^{-7}–10^{-9} M in the presence of HepG2 tumor cells and MRC5 normal cells [18]. The release of antitumor paclitaxel from these polymeric vesicles is controlled by a pH-dependent swelling instead of disaggregation [18, 19]. The pH-sensitive swelling strongly depends on the topology of the copolymer: the DMAEMA units are randomly distributed in the hydrophobic part of the polymersomes enabling the swelling at acidic pH due to the increased electrostatic repulsions.

More in general, physical and chemical properties of polymers in solution strongly depend on their structure, which can be modulated by covalently linked substituents of different solubility [20] or also by copolymerization of comonomers producing a unique back-bone (e.g. MMA and DMAEMA). In addition, if the polymer in solution is a weak polyelectrolyte, properties such as ionization degree can be influenced by different parameters such as polymer conformation and confinement [21], polyelectrolyte concentration, chain rigidity, the formation of intra- and inter-chain charged hydrogen bonds (c-H-bonds) [22] and architecture [23].

Besides, polyelectrolyte adsorption onto charged nanoparticles, and concurrent effects such as spatial partitioning of ions may be influenced by details of the polyelectrolyte structure (linear or star–like) and size [24]. Such an issue can be fundamental in understanding the bactericidal activity of a polymer when in contact with the outer envelope of a bacteria cell [25].

In any case, a control over the architecture and chemical composition of polyelectrolytes is fundamental to produce pH-sensitive polymers with improved performance and reduced cytotoxicity.

To this end, in this work we focused on the synthesis of MMA and DMAEMA copolymers because of their potential applications in different fields such as the interaction to DNA for the formation of polyplexes [13], pH-sensitive systems for drug delivery [18, 19] and formation of inherently bactericidal materials [17, 25]. The synthesis of smart materials, however, is strictly correlated to the control over the chemical composition, so we report the synthesis of MMA/DMAEMA copolymers in different experimental conditions. The composition of the final copolymer, and the reactivity ratios have been estimated to evaluate the relative reactivity of the two comonomers in ARGET ATRP reactions (Activators Regenerated by Electron Transfer for Atom Transfer Radical Polymerization).

It is well known that controlled polymerization processes are required to design copolymers with a well-defined architecture. ATRP (Atom Transfer Radical Polymerization) is one of the most powerful controlled/living radical polymerization techniques: the living nature depends on the onset of a dynamic equilibrium between dormant species and active radical species. ATRP usually requires a transition metal catalyst in its lowest oxidation state, a ligand to complex and solubilize the metal, an alkyl halide as the initiator (R-X) [26, 27]. A variant of ATRP is ARGET ATRP where

the metal is introduced in its highest oxidation state and the active species is continuously regenerated from a reducing agent like glucose, ascorbic acid and the FDA-approved tin (II) 2-ethylhexanoate ($Sn(EH)_2$) [28, 29]. In ARGET ATRP, the amount of metal catalyst can be decreased by thousand times compared to conventional ATRPs and the procedure can tolerate a large excess of reducing agent. This new procedure avoids the deoxygenation of reaction mixtures and simplifies the ATRP process [30]. ARGET ATRP has been successfully applied for the synthesis of homopolymers of styrene, methyl methacrylate, butyl acrylate [28, 31] and DMAEMA [30], and for the copolymerization of styrene and acrylonitrile [32], styrene and methyl acrylate [33], or methyl acrylate with olefins [34].

2 Experimental Section

Copper bromide ($CuBr_2$), 2,2'-bipyridine (bpy), 2–bromoisobutyryl bromide (BMPB), ascorbic acid, Al_2O_3, DMF were purchased by Sigma-Aldrich and used without any further purification. Methylmethacrylate (MMA) and 2(dimethylamino) ethyl methacrylate (DMAEMA) (Sigma-Aldrich) were passed through a column filled with basic alumina prior to use to remove the inhibitors. All manipulations involving air-sensitive compounds were carried out under nitrogen atmosphere using Schlenk techniques.

Stock solutions of ascorbic acid, Cu (II) and bpy were prepared separately dissolving respectively 20 mg, 2.2 mg and 17.1 mg in 10 mL of DMF. The concentrations of the prepared solutions are: $[CuBr_2] = 1 \times 10^{-3}$ M, $[bpy] = 1 \times 10^{-2}$ M and [ascorbic acid] = 1×10^{-2} M. In a typical run, 100 μL of $CuBr_2$ stock solution, 100 μL of bpy stock solution, 0.5–4.5 mL of MMA and DMAEMA (according to the initial feed ratio), 1.2 μL of 2–bromoisobutyryl bromide (BMPB), 100 μL of ascorbic acid stock solution were added in a 50 mL glass flask, under nitrogen atmosphere. After the addition of the last reactant (ascorbic acid), the mixture was thermostated at 60 °C and magnetically stirred. The reaction was stopped with n-hexane. The precipitated copolymers were filtered and dried in vacuum at 40 °C for 12 h.

The number-average molecular weight M_n and polydispersity index ($Đ = M_w/M_n$) were determined by gel permeation chromatography (GPC) using a Waters Breeze GPC system equipped with a refractive index (RI) detector and four Styragel columns (range 1000–1,000,000 Å) in tetrahydrofuran (THF) as eluent at a flow rate of 1.0 mL/min^{-1}. The calibration curve was established with polystyrene standards.

The mole fractions of MMA and DMAEMA in the final copolymer were evaluated through ^1H NMR spectroscopy. NMR spectra were recorded on a Bruker Avance 400 MHz spectrometer at 25 °C. The samples were prepared by introducing 15 mg of copolymer in 0.4 mL of $CDCl_3$ into a tube with an outer diameter of 0.5 mm.

The conversions and the logarithmic concentrations of the monomers in the kinetic plots were evaluated from the yield and chemical composition of the copolymers.

3 Results and Discussion

The synthesis of pH-sensitive copolymers composed of MMA and DMAEMA is illustrated in the reaction scheme of Fig. 1. DMF was employed just to solubilize the metal catalyst, its ligand and the reducing agent; no additional solvent was included in the reaction mixture to reduce as far as possible the presence of organic solvent. Since the amount of metal catalyst is below ppm ($CuBr_2$ = 1 × 10^{-4} mmol), it is unnecessary the removal of the metal from the final copolymer; the purification procedure is thus simplified.

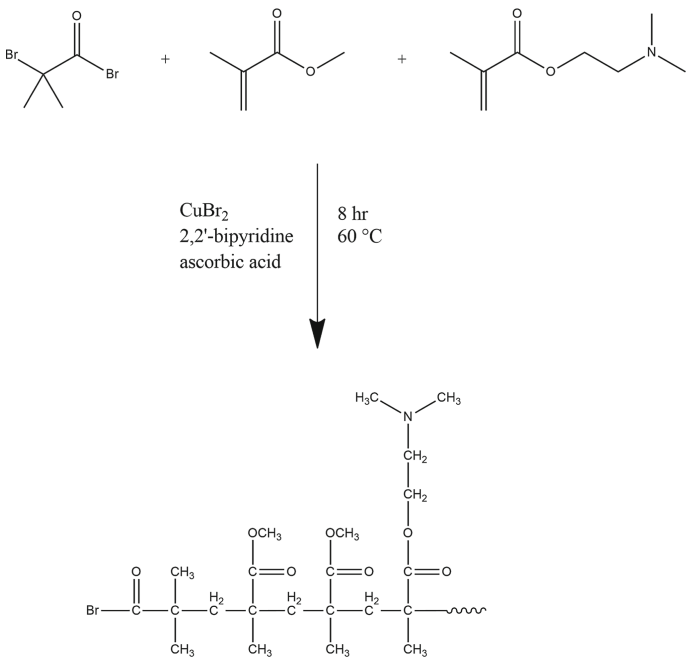

Fig. 1. Reaction scheme for the synthesis of poly(MMA-DMAEMA).

As a first step, the reaction was performed reaching different reaction times to build a kinetic plot. At short polymerization times, the plot of $\ln[M_0/M]$ vs polymerization time shows a downward curvature followed by a linear increase, a typical trend of a slow initiation reaction.

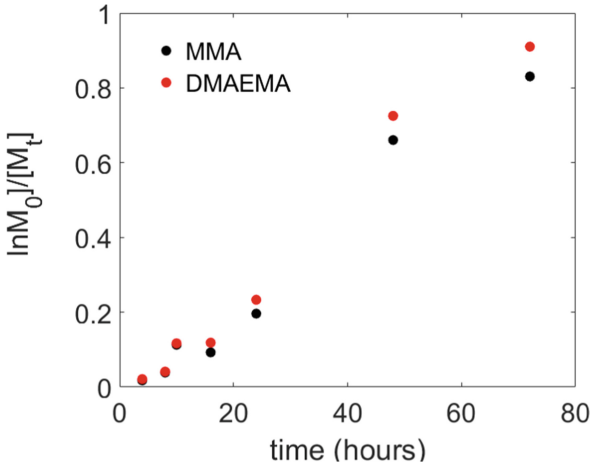

Fig. 2. First order kinetic plot in ARGET ATRP copolymerization of MMA and DMAEMA. Experimental conditions: BMPB initiator, Cu/bpy/BMPB = 1:10:100, $CuBr_2 = 1 \times 10^{-4}$ mmol, $f_{MMA} = 0.61$ and $f_{DMAEMA} = 0.39$. Bulk copolymerization. $V_{tot} \approx 5$ mL, $T_p = 60$ °C.

In order to establish the monomers distribution in the final copolymer, a set of copolymerization runs were carried out changing systematically the molar ratios of MMA and DMAEMA (conditions indicated in Table 1).

Table 1. Copolymerization of MMA and DMAEMA: experimental data used for the evaluation of the final composition[a]

Entry	f^b_{MMA}	F^c_{MMA}	conv. MMA (%)	Yield (g)	M_n (kDa)	$Đ$ (M_w/M_n)
1	0.15	0.13	20	1.0715	37.2	2.7
2	0.29	0.27	22	1.1090	69.2	1.7
3	0.52	0.47	18	0.9243	39.2	1.8
4	0.61	0.60	3.8	0.1834	45.5	1.6
5	0.71	0.67	21	1.0488	26.8	2.3
6	0.86	0.84	2.7	0.1347	20.4	2.0

[a]Experimental conditions: Cu/bpy/BMPB = 1:10:100, $CuBr_2 = 1 \times 10^{-4}$ mmol. Bulk copolymerization. $V_{tot} \approx 5$ mL, $T_p = 60$ °C, $t_p = 8$ h. [b]Mole fraction of MMA in the feed. [c]Mole fraction of MMA in the copolymer.

The copolymer composition was determined from ^{1}H-NMR spectral analysis of the copolymer (Fig. 3) using the Eqs. (1) and (2) [35]:

$$F_{\mathrm{MMA}} = \frac{2I_{\mathrm{MMA}}}{2I_{\mathrm{MMA}} + 3I_{\mathrm{DMAEMA}}} \tag{1}$$

$$F_{\mathrm{DMAEMA}} = \frac{3I_{\mathrm{DMAEMA}}}{2I_{\mathrm{MMA}} + 3I_{\mathrm{DMAEMA}}} \tag{2}$$

where F_{MMA} and F_{DMAEMA} are respectively the molar fractions of MMA and DMAEMA in the final copolymer, I_{DMAEMA} is the integration of the methylene group of the signal relative to DMAEMA units (-O-CH_2-CH_2-N$(CH_3)_2$), labeled as **f** in the NMR spectrum), I_{MMA} is the integration relative to the -OCH_3 unit of MMA (peak labeled as **c** in the NMR spectrum).

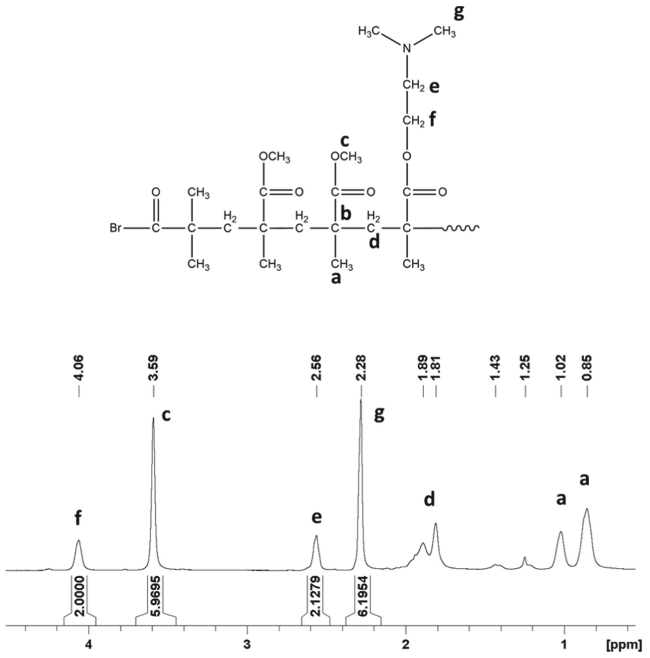

Fig. 3. ^{1}H-NMR spectrum for a MMA/DMAEMA copolymer.

The plot of MMA molar fraction in the final copolymer (F_{MMA}) vs the feed (f_{MMA}) is roughly linear as expected from an ideal copolymerization (Fig. 4).

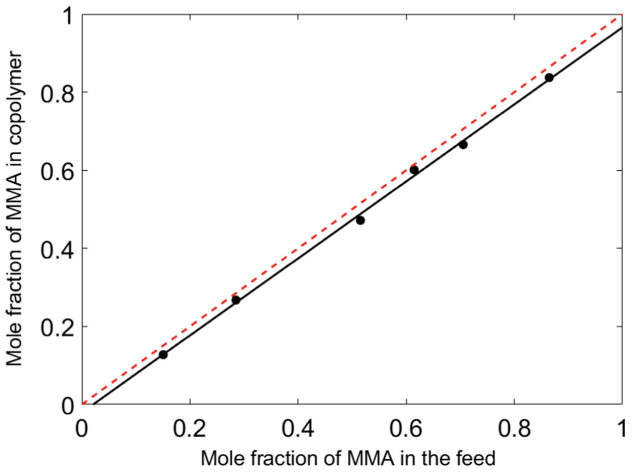

Fig. 4. Plot of MMA mole fraction in the copolymer (F_{MMA}) and in the feed (f_{MMA}). The theoretical curve for an ideal random copolymerization is indicated with a red dashed line.

The monomer feed ratios and the final copolymer compositions of Table 1 were used to estimate the reactivity ratios of the two monomers defined as $r_1 = k_{MMA,MMA}/k_{MMA,DMAEMA}$ and $r_2 = k_{DMAEMA,DMAEMA}/k_{MMA,DMAEMA}$ with $k_{i,j}$ the kinetic rate constants for the reaction between two possible radical sites during the propagation step (scheme in Eqs. 3–6 where M_1 is the monomer MMA, M_2 is the monomer DMAEMA, M_1^* and M_2^* are the reactive terminal units of the growing chain).

$$M_1 + M_1^* \xrightarrow{k_{MMA,MMA}} M_1M_1^* \tag{3}$$

$$M_2 + M_1^* \xrightarrow{k_{MMA,DMAEMA}} M_1M_2^* \tag{4}$$

$$M_2 + M_2^* \xrightarrow{k_{DMAEMA,DMAEMA}} M_2M_2^* \tag{5}$$

$$M_1 + M_2^* \xrightarrow{k_{DMAEMA,MMA}} M_2M_1^* \tag{6}$$

The evaluation of reactivity ratios is helpful for the tuning of the copolymer properties to attain the desired applications (drug delivery systems, coatings [36, 37]). In this work, the reactivity ratios r_1 and r_2 were preliminary calculated with the Mayo-Lewis equation [38] (Eq. 7).

$$F_1 = \frac{r_1 f_1^2 + f_1 f_2}{r_1 f_1^2 + 2 f_1 f_2 + r_2 f_2^2} \tag{7}$$

where $f_1 = f_{MMA}$, $f_2 = f_{DMAEMA}$, $F_1 = F_{MMA}$ and $F_2 = F_{DMAEMA}$, r_1 and r_2 are respectively the reactivity ratios of MMA and DMAEMA.

The Mayo-Lewis equation was rearranged into the linear form proposed by Fineman and Ross [39] (FR, Eq. 8), with slope r_1 and intercept r_2.

$$G = r_1 F - r_2 \tag{8}$$

where $G = x[1-(1/y)]$, $F = x^2/y$, $x = f_1/f_2$, y = F_1/F_2.

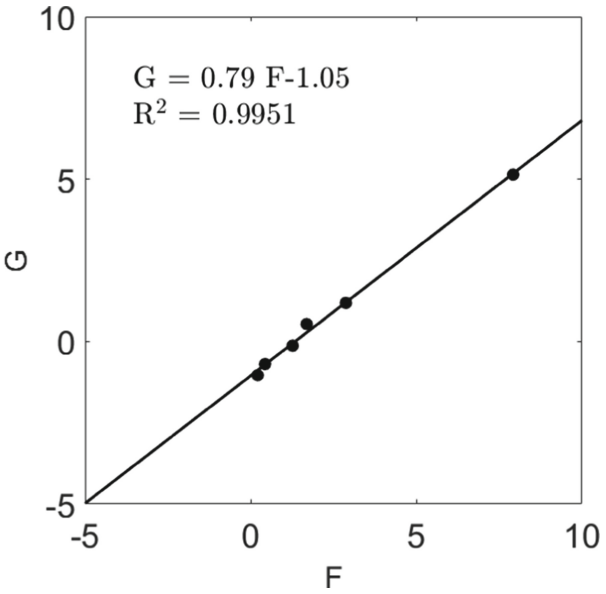

Fig. 5. Fineman-Ross plot for the system poly(MMA-DMAEMA).

The reactivity ratios estimated from the FR plot (Fig. 5) are r_1 = 0.79 (±3%) and r_2 = 1.05 (±8%). However, since most of the conversions reported in Table 1 are higher than 10% and just entry 4 and entry 6 of Table 1 could be strictly used in the Fineman Ross method, the reactivity ratios were also calculated with Eq. (9) that is the integrated form of the Mayo-Lewis model, the Meyer-Lowry equation [40]:

$$\frac{M}{M_0} = \left(\frac{f_1}{(f_1)_0}\right)^{\alpha}\left(\frac{1-f_1}{1-(f_1)_0}\right)^{\beta}\left(\frac{(f_1)_0-\delta}{f_1-\delta}\right)^{\gamma} \tag{9}$$

where M/M_0 is the total monomer conversion, $\alpha = r_2/(1-r_2)$; $\beta = r_1/(1-r_1)$; $\gamma = (1-r_1r_2)/((1-r_1)(1-r_2))$; and $\delta = (1-r_2)/(2-r_1-r_2)$. The reactivity ratios evaluated from the Meyer-Lowry plot, r_1 = 0.76 (±6%) and r_2 = 1.08 (±4%) are in agreement with the Fineman-Ross ratios, thus the monomer conversion does not influence the reactivity between the two monomers as expected from a quasi-ideal copolymerization. The value of r_1 estimated indicates the preference for MMA to react with DMAEMA, whereas the value of r_2 referred to DMAEMA, shows that