# Novel Poly(amidoamine)-Based Hydrogels as Scaffolds for Tissue Engineering

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**Summary:** Poly(amidoamine)s are biocompatible biodegradable polymers, which can be easily functionalized with a number of bioactive and biomimetic compounds. Co-polymerization of these polymers with 4-aminobutyl guanidine (agmatine) leads to an RGD mimicking structure. Hydrogels based on this structure showed an enhanced cell adhesion and could be chemically linked to a glass substrate to create a bioadhesive support for cell growth. Preliminary optimization and cell adhesion tests on Madin-Darby Canine Kidney cells were performed, both on functionalized and non-functionalized structures, with promising results.

**Keywords:** agmatine; biocompatible surfaces; hydrogels; poly(amidoamine); RGD mimic

## Introduction

The advancement of biological sciences and biomaterials has given a fundamental contribution to the development of techniques for dealing with proteins, cells and tissues both in vitro and in vivo. These technologies require reliable support materials with good biocompatibility and cell adhesion, and would greatly profit from new materials that are reliable, disposable and simple to use.[1,2] Among synthetic materials, hydrogels present unrivalled tissue-like properties for interactions with living cells,[3] such as similar water content and permeability to oxygen and metabolites. In principle, synthetic hydrogels, as opposed to naturally derived materials (e.g. gelatin, chitosan etc.) should be most advantageous, giving the possibility of a complete control over hydrogel composition, surface properties and other key parameters such as water absorption and (bio)degradation time. Moreover, hydrogel structures could be used to encapsulate cells, proteins and signaling factors, as well as bioactive moieties to be slowly released during cell growth. Provided cell adhesion is effective, supported or free standing micro-and nano-structured hydrogels could be advantageously exploited for tissue engineering, as matrixes for in vitro cell screening, and for microarray technology.[4]

Cell adhesion on synthetic hydrogels, however, is still an issue for many of these materials, such as poly(hydroxyethyl)metacrylate (PHEMA) or crosslinked poly(ethyleneoxide) (PEG) derivatives.[5,6] A number of chemical and physical modifications have been proposed to overcome this problem, often relying on modification of the synthetic surface with biological or biomimetic moieties, peptides or proteins.[7,8]

Cell adhesion is a complex process, mediated by specific interactions between surface ligands and cell receptors;[9] several studies have shown that the arginin-glycin-aspartic acid (RGD) tripeptide and some of its analogues can interact with adhesion regulating proteins of the integrin family, and promote cell adhesion and spreading.[10–12] Modifications of chemical structures in order to include an RGD or

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RGD-like groups have been proposed for a number of applications where interaction with cells was desired, to enhance adhesion or recognition by cellular receptors.[13–14]

Research in our group has been for several years focused on biomaterials based on poly(amidoamine)s (PAAs). PAAs are synthetic polymers endowed with many biologically interesting properties, being highly biocompatible, non toxic and biodegradable.[15,16] They are obtained by Michael-type addition of bis-acrylamides to primary amines and/or secondary diamines, under mild conditions. Several structures including biologic, biomimetic and bioactive compounds, can be incorporated in the PAA backbone by covalent attachment during the synthetic process.[17–19] Hydrogels based on PAAs besides having both the properties of hydrogels and PAAs, can be easily modified by the introduction of functional co-monomers.[20,21] These extremely versatile materials are especially interesting when carrying carboxylic groups as well as amino groups, since amphoteric PAAs have been demonstrated to have very low toxicity and ''stealth'' properties in vivo.[22]

In the last years, PAA polymers containing RGD mimic units have been prepared. They are obtained by the introduction of 4-aminobutylguanidine (agmatine), used as co-monomer, to build a functional amphoteric repeating unit that is structurally similar to RGD. The soluble polymers carrying these functionalities have extremely low toxicity even if they are polycationic at physiologic pH[23] and have shown potential as transfection agents.[24] Introducing this structure in a crosslinked amphoteric PAA hydrogel resulted in an enhancement of adhesion for fibroblasts.[25] In this work, a similar adhesion promoting material was used to prepare a thin film supported on glass, as a model device for cell screening. Comparative tests of cell adhesion and growth of RGD-binding $\alpha_V\beta_3$ integrin expressing epithelial Madin-Darby canine kidney (MDCK) cells were performed to assess its efficiency, with promising results.[26]

## Materials and Methods

Ethanol, hydrochloric acid (37%), nitric acid (65%), 3-aminopropyltrimethoxy silane, 1,2-diaminoethane (EDA), 4-aminobutylguanidine sulfate (agmatine sulfate), purity 97%, cystamine di-hidrochloride (Fluka, >98%,), and GRGD peptide were purchased from Sigma-Aldrich and used as received. 2-Methylpiperazine was purchased from Fluka and used after sublimation. Its final purity (96,8%) was determined with acidimetric titration. N,N′-Bis (acrylamido)acetic acid (BAC) was prepared as reported in the literature[27] and purity (97,5%) determined by NMR and titration. Phosphate buffer solution (PBS) 10 mM was prepared using Sigma Aldrich tablets according to manufacturer's instructions. All chemicals used in the biological tests were purchased from Sigma-Aldrich. TCPS (tissue culture plate surfaces), multiwells, and tissue culture flasks were purchased from Zellkultur und Labortechnologie, Switzerland; round glass coverslips as support for hydrogels (13 mm in diameter, 0.7 mm thickness) from Zeus super.

Spin coating was performed using a Laurell WS-400B-6NPP –Lite spin coater.

1H and 13CNMRspectra were obtained using a Brüker Avance400 spectrometer operating at 400.132 MHz (1H) and 100.623(13C), and using Brüker software.

*Preparation procedure for AGMA1-75 hydrogel:* in a 10 ml round bottomed flask BAC (1099 mg, 5.4 mmol) was added under nitrogen atmosphere and stirring to an aqueous lithium hydroxide solution ($LiOH^*H_2O$, 226.26 mg 5.4 mmol in 1.8 ml). After complete dissolution, agmatine sulfate (308.17 mg, 1.35 mmol) and more lithium hydroxide monohydrate (81.9 mg, 2.7 mmol) were added and dissolved. This mixture was allowed to react for 24 hr at room temperature ($20 \pm 5\,^{\circ}C$) in the dark, and then EDA (121.7 mg, 2.05 mmol) was added. The solution was stirred for 2 minutes, retrieved with a syringe and injected in a square mould made of two silanized $10 \times 10$ cm glass plates separated

 

by a 0.3 mm silicone spacer. The hydrogel was allowed to crosslink at room temperature for 72 hr, and retrieved as a pliant solid film. The sample was purified by extracting with excess ethanol and then with doubly distilled water. To avoid ruptures caused by osmotic shock, water was not added at once to the ethanol-swollen hydrogel; instead, the sample was exposed to water/ethanol mixtures with increasing water concentrations, until pure water was used. The extraction time was at least 30 min for each step.

ISA23-75 was prepared and purified using the same procedure, and the following reagents: BAC (1099 mg, 5.4 mmol), lithium hydroxide monohydrate (226.26 mg 5.4 mmol), 2-methylpiperazine (135.3 mg, 1.35 mmol), doubly distilled water (1.8 ml), and EDA (121.7 mg, 2.05 mmol). Structure was confirmed by IR spectroscopy in KBr.

Soluble AGMA1 was prepared as reported in literature,[23] its structure confirmed by NMR and GPC. Molecular weight of the sample used: $\overline{M}_n = 5500$ and $\overline{M}_w = 6500$, polydispersity = 1.25; its NMR was consistent with those reported in the literature.

*Glass amino silane functionalization:* Round glass coverslips, 13 mm in diameter, were treated as previously reported.[28] They were soaked in aqua regia at room temperature for 5 hr (20 coverslips were laid out in a glass dish 100 mm in diameter and covered with 12 ml of the acid mixture), washed several times in doubly distilled water and then in ethanol before being soaked in a 10% v/v ethanol solution of 3-aminopropyltrimethoxy silane (15 ml) overnight. The samples were recovered and washed in ethanol ($2 \times 20$ ml), doubly distilled water ($3 \times 20$ ml), and then sonicated in doubly distilled water. They were finally dried with soft paper and used within 24 hr.

*Supported hydrogel layer preparation:* AGMA1-75: BAC (39 mg 0.197 mmol) was dissolved in doubly distilled water (66 μl) together with lithium hydroxide monohydrate (14.5 mg, 0.30 mmol). After the solution cleared, agmatine sulfate (11.20 mg 0.05 mmol) was added and dissolved. The mixture was allowed to react, in the dark and under nitrogen, for 24 hr at room temperature ($20 \pm 5$ °C), then EDA (6.4 mg 0.09 mmol) was added just before casting. About 20 μl of the solution were cast on each pre-treated glass coverslip, using a Pasteur pipette, before spin coating them.

After deposition, samples were kept in a closed sterile container for 3 days at room temperature, to allow the cross linking reaction to proceed. Then they were retrieved, put each in a well of a multiwell plate and washed as described for the free hydrogels, each sample being soaked in 1 ml solution. 30 min after the last addition of water/ethanol mixture, the solution was removed, and replaced with 1 ml of doubly distilled sterile water. Samples were kept in water at 37 °C overnight, rinsed in doubly distilled water and sterilized with UV-rays for ten minutes before use.

ISA23-75: The procedure was the same as reported above for AGMA1-75, using the following quantities: BAC (39 mg, 0.197 mmol) doubly distilled water (66 μl), lithium hydroxide monohydrate (8.25 mg, 0.197 mmol), 2-methylpiperazine (5.0 mg 0.05, mmol), EDA (4.2 mg, 0.68 mmol).

*Cell culture condition:* Immortalized MDCK cells were cultured in Dulbecco's Modified Eagle's Medium, supplemented with 10% Fetal Bovine Serum, 2 mM L-Glutamine, 0.1 mM non essential amino acids, 1.5 g/l sodium bicarbonate, 1 mM sodium pyruvate, 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were grown in tissue culture flasks at 37 °C in controlled atmosphere (5% $CO_2$). After achieving 70% of confluence into the flask, the cells were passed using Trypsin-EDTA solution and seeded at a concentration of 104 cells/well to 13 mm diameter round glass coverslips coated with AGMA1-75, ISA23-75 and to TCPS.

*Cell adhesion, proliferation and morphology:* We measured MDCK adhesion on AGMA1-75, ISA23-75 and TCPS and compared the results. The seeded cells were stored into a humidified incubator at 37 °C. They were monitored every 30 min

 

during the first four hours after cell plating, then every hour for the next 2 or 3 hr. Afterwards they were observed once a day until cells achieved confluence. Images from each sample were collected with a Power Shot G6 Canon digital camera mounted on a Zeiss Axiovert 40CFL inverted optical microscope using 10× objective lens. Four random fields from each sample were photographed and the number of cells both adhered and not adhered was counted to determine the percentage of adhesion, defined as:

$$\text{Adhesion}\ (\%) = \left(\begin{array}{c}\text{number of adhered cells}/\\ \text{total number of cells}\end{array}\right) \times 100.$$

For the cell adhesion experiments in the presence of soluble AGMA1 or GRGD peptide, cells were seeded in culture medium supplemented with 1 mM AGMA1 (calculated on the repeating unit concentration), 10 mM AGMA1 or 1 mM GRGD. After 4 hr we calculated the inhibition of adhesion defined as:

$$\text{Inhibition}\ (\%) = [1 - (\text{Adhesion}(\%)/\text{Adhesion}\ (\%)\ \text{in control condition})] \times 100,$$

where Adhesion (%) in control condition is the result of cell adhesion on each substrate (TCPS, AGMA1-75 and ISA23-75) in medium without soluble AGMA1 or GRGD.

## Results and Discussion

Previous papers had reported on the effect of agmatine-functionalized hydrogels on the adhesion of fibroblasts (Figure 1).

In our case, we decided to exploit the same functionalization to enhance the hydrogel adhesion of MDCK epithelial cells, in order to prepare a prototype supported device. The presence of a glass support would greatly enhance the hydrogel usefulness in the field of cell screening and molecular diagnostics.

For this reason we have designed, prepared and tested a new bi-layered system. This new system is composed by a glass support covered with a functional hydrogel layer. The glass substrate provides mechanically robust support and easier handling while the functionalized hydrogel layer interacts with the cells. Both glass support and hydrogel are optically transparent, as required for several cell characterization techniques, including optical microscopy.

As pointed out in the introduction, in this study we chose to compare hydrogels based on two amphoteric PAA structures, AGMA1 and ISA23, both known in the literature as highly biocompatible

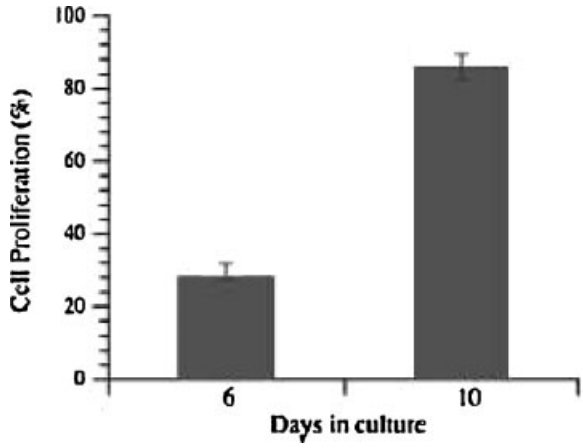


***Figure 1.***
Effect of Agmatine functionalization on cell adhesion and proliferation for fibroblasts.

 

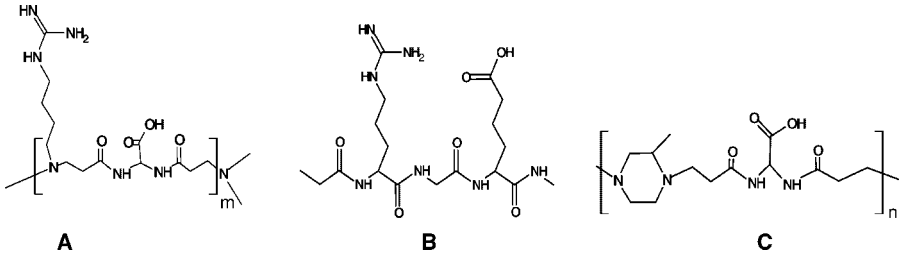


***Figure 2.***
Structure of AGMA1 (A) and ISA23 (C) and the RGD peptide (B).

structures. The AGMA1 repeating units are very similar to the well known adhesion-modulating RGD peptide sequence. Figure 2 shows the repeating unit of AGMA1 polymers (A) and the structure of the RGD peptide (B). ISA23, not carrying any guanidine pendant group (structure reported in Figure 2C), can be used as a non-functional model, to rule out the effect of mechanical properties and overall surface morphology. Linear ISA23 polymers are known to be biocompatible[21,22] and their hydrogels show no significant cell adhesion properties.[29]

A semi-quantitative evaluation of the cell adhesion properties of these systems, in order to evaluate the best composition for cell adhesion was performed with different compositions. Results are reported in Figure 3.

Interestingly, the best composition, which had 19% mol/mol crosslinker, and 12,5% mol/mol agmatine content has a composition similar to that of the samples found to be effective in the fibroblast tests (16,5% mol/mol crosslinker content, 8,3% agmatine).

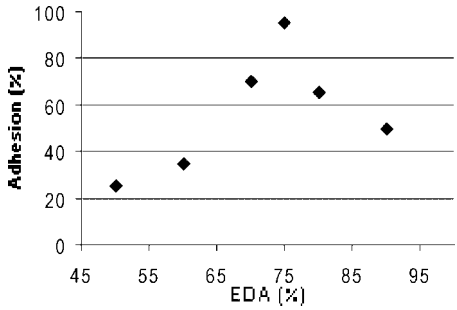


***Figure 3.***
Cell adhesion behavior (normalized against TCPS) versus crosslinker content, given as moles ratio, that is (moles of EDA aminic hydrogens /moles of overall aminic hydrogens) × 100.

*Cell adhesion:* After seeding 10000 cells in each well, the optical microscope showed round and pearly cells until they began to attach to the substrates. Up to 2 hr, cell adhesion on AGMA1-75 and ISA23-75 was not markedly different and adherent cells on both substrates are much less than on TCPS. At 4 hr cells on ISA23-75 were still few and showed the same morphology, whereas on AGMA1-75 the number of cells adhered to the substrate was similar to that on TCPS. At longer times, cells on TCPS start to proliferate and form clusters. On AGMA1-75, instead, cells proliferated slowly. This behavior becomes even more evident at 72 hr when cells seeded on TCPS have achieved confluence and begin to die. On AGMA1-75, instead, we still observe cell clusters without confluence. This effect may be explained considering that despite the fact that PAA hydrogel layer were supported by a rigid material, cells probably experienced a more compliant substrate than TCPS. It generally recognized that focal contact formation and cytoskeletal assembly may be relatively more difficult on hydrogel surface, retarding cell growth.[30]

MDCK cells grown on ISA23-75 exhibited lower adhesion and slower proliferation than the AGMA1-75 and TCPS grown cells. However, after 48 hr, cells attached to some extent also on this substrate. This effect might be explained by the partial absorption of adhesive proteins from serum on to the hydrogel.

 

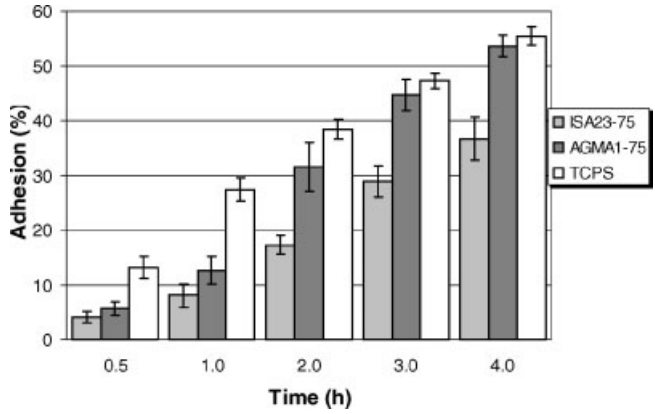


***Figure 4.***
Cell adhesion vs time on the substrates examined: ISA23-75, AGMA1-75 and TCPS. The percentage of cell adhesion on ISA23-75 and AGMA1-75, is comparable in the first hour, and much lower than the adhesion on TCPS. After two hrs cell adhesion percentage on AGMA1-75 is higher than on ISA23-75 and similar to the value on TCPS.

A quantitative evaluation of MDCK adhesion on substrates as a function of time showed that the adhesion had a quadratic trend on TCPS and AGMA1-75 whereas a linear trend on ISA23-75, as reported in Figure 4. The histogram shows that, up to 2 hr after seeding, cell adhesion on TCPS is higher than on hydrogels, while between 2 and 4 hr MDCK adhesion on AGMA1-75 increases remarkably compared to ISA23-75 until reaching an adhesion percentage similar to that on TCPS.

*Soluble AGMA-1 and GRGD similarly prevent cell-substrate adhesion:* In order to confirm that the difference in cell adhesion on the Agmatine-functionalized hydrogels is due to its composition and not to other factors, such as non-specific serum protein adsorption, we tested the adhesion-inhibiting effect of soluble AGMA1 polymers and GRGD peptides.

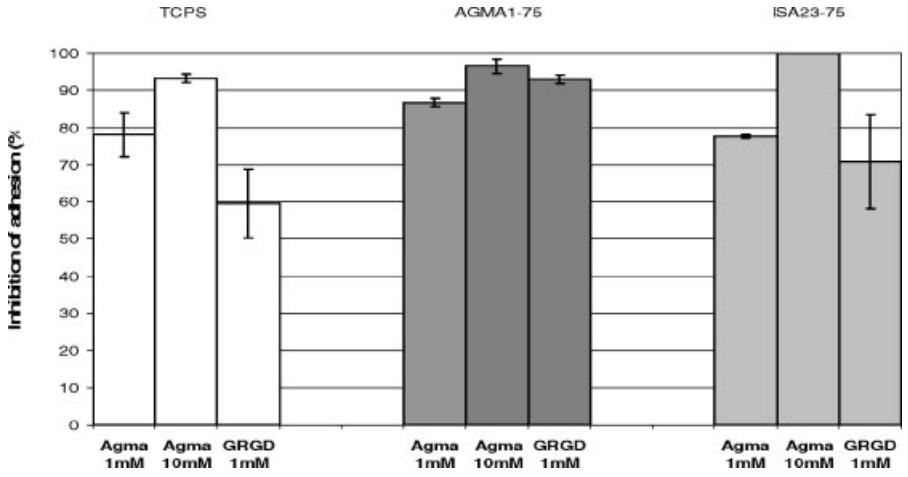


***Figure 5.***
The presence of a soluble polymer bearing the agmatine-BAC sequence is able to prevent cell adhesion on all the substrates. 1 mM GRGD peptide and 1 mM AGMA1 (calculated on repeating unit concentration) have the same effect on cell adhesion inhibition; increasing the soluble polymer concentration to 10 mM does not increase significantly the inhibition of cell adhesion.

 

The presence in the medium of a soluble polymer obtained by copolymerization of BAC and agmatine[23] up to a concentration of 1 mM in repeating units, proved to prevent cell adhesion on all the substrates (Figure 5).

Increasing the AGMA1 concentration up to 10 mM did not significantly increase the inhibition of cell adhesion, suggesting that the interested receptors are already almost completely saturated at 1 mM AGMA1. Since 1 mM GRGD peptide and 1 mM AGMA1 (calculated on repeating unit concentration) have the same effect on cell adhesion inhibition, it can be reasonably concluded that both compounds bind the same integrin receptors.

## Conclusions

Several possible applications can be envisioned for agmatine-functionalized PAA hydrogels. These results are a further step in this direction, showing how the cell-polymer interaction can be controlled by a simple chemical modification. Interaction with the cell receptors was confirmed, as well as biocompatibility, and a cell adhesion as good as that on TCPS for the epithelial MDCK cells.

Extending of these results on to other cell lines as well as further studies in the mechanics of the cell-AGMA1 interactions are ongoing. The growth retarding effect of the hydrogel surface can also be interesting in order to apply these substrates to conservation and proliferation of stem cells.

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